THE UNIVERSITY OF ALBERTA

T CELL REPERTOIRE TO THE SYNTHETIC POLYPEPTIDE ANTIGEN POLY-18

by

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T cell repertoire to the synthetic
polypeptide antigen Poly-18

submitted by Zuzana Novak
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DEDICATION

To Philip W. Spink

You are
my wish upon the stars above
thanks for being here
you fill each moment of
my life

with love

ABSTRACT

We analyzed the expressed T cell repertoire to the immune response (Ir) gene controlled synthetic antigen Poly-18, Poly-EYK(EYA)$_5$, in responder BALB/c (H-2^d) mice. Using an extensive panel of Poly-18 related peptides and Poly-18 specific normal T cells and T cell hybridomas we determined that even though it is a repeating antigen and all of its epitopes have extensive amino acid sequence overlap, the immune reponse to Poly-18 is focused only onto the epitopes EYK(EYA)$_4$ and (EYA)$_5$. These epitopes are strongly immunogenic and antigenic and are recognized by I-A^d restricted, Poly-18 specific, Type A and Type B T cell clones, respectively. The T cell response directed at the (EYA)$_5$ epitope is extremely heterogeneous even though the epitope has a relatively simple amino acid sequence. Some Poly-18 epitopes elicit T cell responses only when a peptide representing that epitope is used for immunization, indicating that the repertoire is larger than that detected by immunization with the whole Poly-18 molecule and that antigen processing plays a role in clonotype selection.

We also examined whether T cell genotype contributes to the selection of the expressed T cell repertoire by analyzing the full range of I-A^d restricted Poly-18 specificities generated by responder H-2^{bxd} and nonresponder H-2^b T cells which were educated in a H-2^{bxd} responder thymic environment. We found that unlike normal H-2^d, H-2^{bxd}F1 and chimeric H-2^{bxd}F1->H-2^{bxd}F1 animals, the H-2^b->H-2^{bxd}F1 chimeric animals only generate Type B T cell clones in response to Poly-18. Such restrictions in the expressed T cell repertoire could be related to genotype

specific preferences in T cell receptor V gene usage as well as H-2^b specific regulatory/deletion mechanisms. Alternatively, Type A and Type B T cell clonotypes may be differentially sensitive to the limiting numbers of responder H-2^{bxd} APC in the H-2^b->H-2^{bxd}F1 priming environment.

In conclusion, the diversity of the T cell response to a repeating antigen such as Poly-18 provided us with a glimpse of the complexity and diversity of the immune system.

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ABBREVIATIONS

APC	antigen presenting cell(s)
ATS	anti-thymocyte serum
B cell	bone marrow derived lymphocyte
CFA	complete Freund's adjuvant
CTL	cytotoxic T lymphocyte(s)
DC	dendritic cell(s)
dGuo	deoxyguanosine
DNP	dinitrophenyl
DTH	delayed-type hypersensitivity
FITC	fluorescein isothiocyanate
GAT	poly-(L-glutamic acid^{60}, L-alanine^{30}, L-tyrosine^{10})
GL	poly-(L-glutamic acid, L-lysine)
GT	poly-(L-glutamic acid, L-tyrosine)
H	high
H-2	mouse major histocompatibility complex
HA	hemeagglutinin
HEL	hen egg lysozyme
HLA	human major histocompatibility complex
HR	high responder
H(Thy)	high responder thymus
H-Y	male specific antigen
Ia	I region encoded antigen
Ig	immunoglobulin
Ir gene	immune response gene
irr	irradiation

KLH	keyhole limpet hemocyanin
L	low
LR	low responder
L(Thy)	low responder thymus
MHC	major histocompatibility complex
minor HA	minor histocompatibility antigen
MLR	mixed lymphocyte reaction
Mls	minor lymphocyte stimulatory locus
NR	non responder
nu	nude
PLL	poly-L-lysine
Poly-18	poly-EYK(EYA)$_5$ where (E) L-glutamic acid, (Y) L-tyrosine, (K) L-lysine, (A) L-alanine
R	1. responder 2. rads -when prefixed with numbers
REL	ring-necked pheasant lysozyme
SD	standard deviation
SRBC	sheep red blood cells
SWM	sperm whale myoglobin
T cell	thymus derived lymphocyte
TcR	T cell receptor
(T,G)-A--L	poly-(L-tyrosine, L-glutamic acid)-poly-D,L-alanine--poly-L-lysine
Th	helper T lymphocyte(s)
TNP	trinitrophenyl
VSV	vesicular stomatitis virus

CHAPTER 1

Introduction

The two arms of the immune system, the humoral mediated by B lymphocytes, and the the cellular, mediated by T lymphocytes, utilize very diverse repertoires of antigen specific receptors for the recognition of foreign antigenic molecules. These foreign molecules are frequently proteins and include antigens of infectious and parasitic organisms, allergens as well as transplantation antigens. The antigen specific receptors of B cells and T cells, referred to as immunoglobulins(Ig) and T cell receptors(TcR) respectively, are clonally expressed cell surface structures composed of glycosylated polypeptide chains which have constant COOH-terminal domains and highly variable NH_2-terminal domains making up their antigen binding sites [1,2]. Although the antigen specific receptors of both B cells and T cells contain highly variable antigen binding sites the mechanisms of antigen recognition by B cells and T cells are quite different. Immunoglobulin molecules specific for native proteins can bind free antigen in solution while TcR's recognize antigens only on the surface of antigen presenting cells (APC) [3]. Furthermore, T cells recognize antigen only after it has been "processed", i.e. denatured and/or fragmented, by the antigen presenting cells [4]. As a result, T cells do not generally distinguish between native or denatured or fragmented forms of protein antigens [5] whereas immunoglobulin recognition generally does depend on the integrity of the tertiary structure of native protein antigens[6]. Finally, the processed antigen is recognized by TcR's on the suface of antigen presenting cells only in association with cell surface molecules encoded by the major histocompatibility complex(MHC) [7]. Antigens which associate with MHC Class I molecules are generally recognized by cytotoxic T lymphocytes(CTL) while antigens which associate with MHC Class II molecules are generally recognized by helper T lymphocytes (Th) [8]. Due to the MHC restricted recognition of antigen by T cells, the selection of the antigen specific T cell repertoire in the thymus as well as the subsequent triggering of the selected T cell clones in the periphery is strongly influenced by the expressed MHC molecules.

T cell dependent immune responses are MHC restricted

MHC restriction is an experimental observation whereby antigen specific T cells interact with other cells such as B cells, T cells and macrophages only if they are histocompatible with the antigen specific T cells. Rosenthal & Shevach [9] demonstrated that MHC identity between T cells and macrophages was obligatory for the antigen specific proliferation of T cells. This effect was later mapped in the mouse to the I-A region of the MHC gene complex [10]. At the same time Katz et al.[11] showed that T cells and B cells must be histocompatible to cooperate in antibody production and that this effect also maps to the I-A region of the mouse MHC [12]. Similarly, to achieve successful transfer of delayed type hypersensitivity (DTH), there is a requirement for I-A matching between donors of sensitized DTH T cells and naive recipients [13]. By contrast, antigen specific cytotoxic T cells (CTL) were shown to be restricted by the K, D and L regions of the mouse MHC. Zinkernagel & Doherty [14] demonstrated that target cells and virus specific CTL must be histocompatible for target lysis to occur while Shearer et al.[15] mapped this effect to the K and D regions of the mouse MHC using trinitrophenyl(TNP) modified target cells and TNP specific CTL. Finally, when MHC restriction was observed with T suppressor cells, it frequently mapped to the I-J region of the mouse MHC [16].

Immune responsiveness maps to the MHC gene locus

In addition to the restrictive elements summarized above, the MHC codes for genes which govern the strength of T cell dependent immune responses [17]. DTH and antibody responses to simple amino acid polymers such as (DNP-PLL) and (GL) were found by Benacerraf et al.[18] to occur in some outbred guinea pigs but not others. Experiments with inbred guinea pig strains indicated that this responsiveness was determined by a single genetic locus [19,20]. McDevitt et al.[21] subsequently mapped these immune response (Ir) genes, responsible for determining the responder status of mice to a series of branch chain amino acid polymers, to a region of the mouse MHC which he designated as the I-region. Ir-genes were subsequently shown to express their functions in cells which collaborate with T cell. Thus, Katz et al.[22] showed that antigen specific T cells from (nonresponder x responder)F1 donors cooperate with B cells from responder(R) but not nonresponder(NR) donors in antibody production. Similarly, antigen specific T cells from (NR x R)F1 mice proliferate to antigen presented on R but not NR macrophages [23,24]. Ir gene controlled responses were also observed in CTL but this time they mapped to the K or D regions of the mouse MHC. It was observed that some viruses could only be recognized in the context of some but not all Class I MHC restricting elements [25-27].

Class I and Class II MHC molecules are the restriction elements for T cells

The parallels between MHC restriction and Ir-gene control are not coincidental. Class I and Class II MHC molecules [28] have been demonstrated to be both the restriction elements for antigen specific T cells as well as the gene products of Ir-gene loci.

Alloantisera generated against lymphocyte alloantigens [29-31] as well as alloantisera generated against Ia determinants [32,33] have been shown to inhibit self MHC restricted T cell mediated immune responses in vitro, as have monoclonal antibodies directed at specific Ir gene products [34,35].

Experiments with mice bearing mutant Class I H-2K^b molecules ie. B6.C(H-2^{bm1}) and (H-2^{bm6}) mice, or Class II ie. B6.C(H-2^{bm12}) which bears a mutant Aβ chain of the I-A^b molecule, indicate that Th cells or CTL cells which are specific for antigen in the context of normal I-A^b [36] or K^b [37], respectively, will not respond to antigen in the context of the mutant MHC molecules. Similarly, the expression of normal I-A^b or mutant I-A^b molecules alters the Ir-gene responder status of otherwise genetically identical mice. C57BL/6 mice bear normal I-A^b molecules and are responders to beef insulin and nonresponders to poly(GT) while B6.C(H-2^{bm12}) mice bear mutant I-A^b molecules and are nonresponders to beef insulin but responders to poly (GT) [38].

Transfection experiments with cloned Class I genes show that vesicular stomatitis virus (VSV) infected mouse L cells (H-2^k) which were transfected with the H-2L^d gene become targets for VSV specific CTL cells derived from H-2^d bearing mouse strains [39]. This is unequivocal evidence that the H-2L^d gene product is specifically recognized by H-2L^d restricted CTL cells. Similar observations have been made with Class II gene transfections. B lymphoma cells transfected with Aα^b and Aβ^b or with Aα^b and Aβ^{bm12} genes have been shown to be potent I region restricted antigen presenting cells for a large panel of antigen specific, autoreactive and alloreactive T cell hybridomas as well as T cell clones [40,41].

Finally, a number of laboratories have shown that supported planar membranes or liposomes containing purified I-A molecules [42-44] can activate antigen specific Th clones in the presence of antigen. Similarly, purified H-2K^k molecules [45,46] can activate H-2K^k specific CTL cells in vitro.

T cells corecognize antigen and MHC

MHC restricted T cell recognition was thought to involve like-like interactions between histocompatible molecules present on antigen specific Th cells and B cells or macrophages as well as antigen specific CTL cells and their targets. Working on virus induced CTL cells, Zinkernagel & Doherty were the first to speculate that the antigen specific T cell receptor (TcR) recognizes modifications in the MHC molecules that are induced by the viral proteins during viral replication [47]. The recognition of altered self MHC [48] or a self MHC-antigen complex [49,50] by a single TcR has gained favor over other T cell recognition models which propose a separate recognition of antigen and MHC either by two separate receptors [51,52] or one receptor with 2 separate combining sites [53].

The corecognition of MHC-antigen complexes or altered self MHC is supported by experiments using Th cells as well as CTL cells and demonstrating that the antigen- and MHC- specific components do not segregate independently. Kappler et al.[54] fused a T cell hybridoma, specific for ovalbumin in association with $A\beta^k$:$A\alpha^k$ Ia molecules , to a normal T cell line, specific for keyhole limpet hemocyanin (KLH) in association with $A\beta^f$:$A\alpha^f$ Ia molecules. These hybrids could be stimulated only by the original pairs of antigen and Ia molecules suggesting that the recognition of antigen and MHC is not independent. Similarly, analysis of CTL clones with the unusual dual specificity of H-2^k plus a DBA minor antigen and H-2^d plus a BALB minor antigen indicated that such clones could not respond to H-2^k plus a BALB minor antigen or H-2^d plus a DBA minor antigen [49].

The one receptor model is supported by the demonstration that the TcR is a single heterodimeric glycoprotein composed of disulfide linked α and β chains [2]. Transfection experiments clearly indicate that the α and β chains of the TcR are both cruicial and sufficient to transfer both antigen and MHC specificity of the donor T cell [55,56]. However, CD4 and CD8 accessory molecules which are expressed on Class II and Class I restricted T cells respectively [8,57,58], bind to nonpolymorphic portions of Class II and Class I molecules and have been shown to enhance the binding of the TcR to its ligand [59,60]. It is not yet clear whether the binding of CD4 and CD8 molecules to MHC antigens contributes to the signals leading to T cell activation.

Role of the thymus in T cell repertoire selection

Thymocytes can be divided into four subsets based on the expression of the accessory molecules CD4 and CD8, namely $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$ and $CD4^-CD8^+$ [61]. Although various approaches have shown that $CD4^-CD8^-$ double negative thymocytes act as stem cells for the other thymocyte subpopulations [62-64] it is not yet clear whether mature $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells are derived directly from $CD4^-CD8^-$ cells or proceed through an intermediate double positive $CD4^+CD8^+$ stage. Although the turnover of cells in the thymus is very large, 10^8/day in mice, intrathymic labelling studies of thymocytes with FITC indicates that only a very small number of T cells, 1-2 x 10^6/day, ever leave the thymus and that most thymocytes die in situ [65]. This massive cell death occuring in the thymus is most likely related to the selection process imposed upon developing T cells which ensures that the expressed T cell repertoire is both self MHC restricted as well as self tolerant. Since only single positive $CD4^+CD8^-$ and $CD4^-CD8^+$ cells and double positive $CD4^+CD8^+$ cells usually express TcR αβ heterodimers, although double positive cells mostly at low levels [66,67], it is very likely that MHC specific selection of thymocytes occurs at these double or single positive stages of their development. However, a subpopulation of double negative $CD4^-CD8^-$ thymocytes, which generally express TcR γδ receptors, have also been shown to express CD3-associated TcR αβ receptors [68]. During ontogeny in the thymus, the expression of TcR γδ bearing thymocytes has been shown to precede the expression of classical TcR αβ bearing thymocytes [69] but the two types of T cells are not in the same developmental lineage. Analysis of TcR α chain producing T cells indicates that the majority of these cells have never rearranged their TcR δ chain locus and have therefore never expressed the γδ TcR [70].

Thymic selection of MHC restricted T cells

The discovery of MHC restriction sparked intense interest in the issue of how T cells become imprinted with MHC restricted specificity. Experimental evidence suggests that the repertoire of mature T cells is heavily influenced by the microenvironment encountered in the thymus during early T cell development [71]. The experiments of von Boehmer et al. [72] demonstrated that irradiated F1 mice reconstituted with T depleted bone marrow cells of both parental strains, A+B->(AxB)F1 (irr) chimeras, gave rise to parental strain T cells capable of collaborating with B cells of either parental strain. Two hypotheses were forwarded to explain this apparent contradiction of classical MHC restriction. The first hypothesis suggested that normal unprimed T cells may be a mixture of self and allo MHC restricted cells and that the in vivo priming event selectively expands the self MHC restricted cells due to the presence of self antigen presenting cells ie. macrophages while the second hypothesis put the emphasis on the environment rather that the antigen priming event. It was proposed that T cells differentiating in an MHC different environment might undergo some process of "adaptation" which would result in their ablity to recognize their developmental environment as self and thereby enable them to interact with MHC different B cells [73].

Adaptive differentiation of Class I restricted T cells

The first experiment to support the adaptive differentiation hypothesis was that of Bevan [74] demonstrating that T cell depleted F1 bone marrow cells transferred into heavily irradiated parental strain mice, (AxB)F1->A (irr) chimeras, generated a population of F1 T cells which would predominantly lyse strain A target cells bearing appropriate minor histocompatibility antigens (HA) but not strain B target cells bearing appropriate minor HA. Since the lymphohemopoietic system in F1->parent chimeras is almost entirely of donor origin, the T cell repertoire skewing was postulated to involve some radioresistant component of the host, such as the thymus. The idea that thymic elements imprint T cells with H-2 restricted specificity was supported by the experiments of Fink & Bevan [75] using CTL cells specific for minor HA as well as Zinkernagel et al.[52,76] using CTL cells specific for viral antigens. These investigators showed that thymectomized (AxB)F1 mice reconstituted with (AxB)F1 bone marrow cells and then grafted with strain A thymuses developed mostly A restricted CTL cells but not B restricted CTL cells.

Unlike these initial experiments however, Matzinger & Mirkwood [77] showed that CTL cells specific for minor HA raised in fully allogeneic A->B chimeras were restricted to both host and donor MHC. It was also shown by others that thymectomized (AxB)F1 mice reconstituted with strain A bone marrow and grafted with strains A thymuses contained considerable numbers of B restricted CTL cells [78]. Furthermore, CTL cells from nude mice grafted with MHC different thymuses are often completely restricted to host MHC [79] even when the CTL cells are primed to antigen or virus in the context of thymic MHC [80]. In order to explain some of these anomalous results Kruisbeek et al. [81] suggested that CTL cell differentiation may involve an extrathymic pathway in addition to the conventional pathway of maturation in the thymus.

Adaptive differentiation of Class II restricted T cells

In contrast to the results on Class I MHC restriction, Singer,Kruisbeek et al. [81-84] demonstrated in a wide variety of experimental models that Class II restricted T cells show complete restriction to thymic MHC determinants. Although others [85] have generated some evidence to the contrary, it is not very compelling. Several Ir-gene controlled antigen systems have also been used to study the imprinting of MHC restricted specificity in T cells [86].

Kappler & Marrack [87] studied high (H) and low (L) responder mice to the synthetic antigen (T,G)-A-L. They find that L responder T cells derived from L responder plus H responder bone marrow reconstituted (L x H)F1 lethally irradiated recipients, L+H->(L x H)F1 (irr) chimeras, can cooperate with H responder B cells in antibody production. They also constructed L->(L x H)F1 (irr) chimeras and obtained the came result. This time however, they had to inject 10^8 T depleted F1 spleen cells into the chimeras at the time of antigen priming to provide a source of H responder APC. Fully allogeneic chimeras were also investigated using the (T,G)-A-L antigen. Singer et al.[82] generated responder (R)->nonresponder (NR) (irr) as well as NR->R (irr) bone marrow radiation chimeras. Macrophage depleted spleens of these animals were later tested in the presence of (R x NR)F1 splenic adherent cells as APC and TNP-KLH or TNP-(T,G)-A-L for their ability to generate primary anti-TNP antibody responses. Although both types of chimeras responded to TNP-KLH only NR->R (irr) chimeric cells responded to TNP-(T,G)-A-L. These experiments clearly demonstrated that a radiation resistant component of the host was responsible for Class II MHC restricted T cell selection.

To confirm the importance of the thymus in the selection of Class II restricted T cells, Miller et al.[88] studied DTH in thymic grafted mice of responder (R) and nonresponder (NR) phenotype for the synthetic antigen GAT. Thymectomized and irradiated (R x NR)F1 mice were reconstituted with NR bone marrow and a R thymus graft. When these chimeras were sensitized with GAT on (R x NR)F1 peritoneal exudate macrophages the primed NR T cells could mount GAT specific DTH responses just like R T cells. Thymus graft experiments were also performed in nude mice by Hedrick & Watson[89] using high (H) and low (L) responder mice for calf skin collagen. (H x L)F1 nude mice were grafted with H or L responder thymuses and later immunized with collagen or sheep red blood cells(SRBC). Both H(Thy)->(H x L)F1 nu and L(Thy)->(H x L)F1 nu were shown to produce antibodies to SRBC but only H(Thy)->(H x L)F1 nu produced high antibody titres to collagen.

Using an Ir gene controlled antigen system where both Class I and Class II restricted T cell responses were monitored, Kast et al.[90] demonstrated that the radioresistant components of the thymus dictates MHC specificity and immune response phenotype of T cells restricted to Class II (H-2 I-A^b) MHC molecules but not of T cells restricted to Class I (H-2K^b) MHC molecules. Sendai virus nonresponder bm1 (H-2K^b mutant) CTL cells maturing in B6 (H-2K^b) responder as well as bm1 mutant nonresponder thymus grafts in thymectomized (B6 x bm1)F1 responder mice, exhibit B6 restricted Sendai virus specific CTL activity. This is in agreement with Kruisbeek et al.[81] suggesting an extrathymic differentiation pathway for Class I restricted CTL cells. In contrast, H-Y antigen nonresponder bm12 (H-2 I-A^b mutant) CTL cells maturing in a B6 (H-2 I-A^b) responder but not a bm12 nonresponder thymus graft in thymectomized (B6 x bm12)F1 responder mice, exhibit B6 restricted H-Y specific CTL activity.

The T cell receptor and positive thymic selection

Recent experiments provide compelling evidence for the positive selection of T cells by thymic MHC antigens in the absence of nominal antigen. Some investigators have taken advantage of the natural correlation between a particular Vβ gene segment and TcR specificity in order to determine whether the αβ TcR is involved in the thymic selection process. The TcR Vβ6 gene confers preferential reactivity to the Mls antigen, Mls-1^a, which is encoded by the minor lymphocyte stimulatory (Mls) locus of the mouse [91], in association with some MHC Class II determinants ie. H-2^k or H-2^d but not others ie. H-2^q [92]. MacDonald et al.[93] determined that Mls-1^b expressing mouse strains of H-2^k or H-2^d haplotypes generate 2-3 fold higher levels of Vβ6$^+$ CD4$^+$CD8$^-$ T cells than Mls-1^b expressing mouse strains of the H-2^q haplotype. These results suggest that contact with Class II molecules in H-2^d or H-2^k haplotypes in the absence of antigen (Mls-1^a) resulted in the overexpression of Vβ6$^+$ CD4$^+$CD8$^-$ T cells in these mice.

A number of other labs have used transgenic mice, expressing various $\alpha\beta$ TcR transgenes, to study this same question. Sha et al.[94] report that H-2^b transgenic mice expressing the $\alpha\beta$ TcR derived from an H-2^b CTL specific for the Class I molecule H-$2L^d$ express this TcR transgene on 20-90% of their peripheral T cells, most of which are $CD4^-CD8^+$ like the original CTL clone, while H-2^s transgenic mice do not express this TcR transgene on many $CD8^+$ peripheral T cells [95] suggesting that H-2^b MHC molecules are important in expanding this T cell population. Identical conclusions were drawn by Teh et al.[96] with transgenic mice expressing an $\alpha\beta$ TcR derived from an H-2^b CTL specific for the male antigen H-Y in the context of Class I H-$2D^b$ molecules. These investigators observe an elevated proportion of $CD4^-CD8^+$ thymocytes in H-2^b but not in H-2^k or H-2^d thymuses of female mice repopulated by the progeny of $\alpha\beta$ transgenic stem cells. Furthermore, Kisielow, Teh et al.[97] demonstrate that the positive selection of H-$2D^b$ restricted H-Y specific T cells in the thymuses of female mice is strictly dependent on the specific interaction of the $\alpha\beta$ TcR with D^b MHC molecules in the absence of antigen and that this interaction cannot occur with Class I K^k, D^k, K^d, D^d or K^b molecules.

Cell types in thymus controlling positive thymic selection

The issue of which cells imprint MHC-restricted specificity is still unsettled. Longo and co-workers have generated evidence in support of the view that bone marrow derived cells restrict Class II specific T cells. Longo & Schwartz [98] have shown that F1-> parent (900R) chimeras treated with anti-thymocyte serum(ATS) several months later, repopulate the periphery with T cells restricted to both parental strains. In addition , the ATS treatment was not even necessary when chimeras were generated with 1200R irradiation [99]. Finally, Longo et al.[100] observed that (A x B)F1 nude mice grafted with thymuses which contain strain A thymic epithelial but strain B bone marrow cells obtained from long term B->A chimeras preferentially generate B restricted T cells.

In contrast, several investigators have generated evidence in support of the view that radioresistant epithelial cells restrict Class II specific T cells. Ron et al.[101] demonstrate that (A x B)F1-> A parent (1000R) chimeras generate A parent restricted T cells and that subsequent reirradiation with 800R to remove mature T cells from the periphery results in the generation of a new wave of A parent restricted T cells. Similarly, Lo & Sprent [102] observe restriction to thymic MHC determinants in thymectomized F1 mice reconstituted with F1 bone marrow cells and macrophage / dendritic cell(DC) depleted thymus grafts. Although only additional data will resolve this issue, the data that MHC-restriction specificity is determined by radiation resistant epithelial cells is quite strong.

Thymic selection of self MHC tolerant T cells

The peripherally expressed T cell repertoire is restricted to self MHC gene products and yet does not generally respond to self MHC molecules in the absence of exogenous antigen. Bevan & Hunig [103] have demonstrated that T cells preferentially respond to antigens that are similar to self MHC. The high proportion of allo reactive T cells [104] in the peripheral T cell repertoire could thus be related to similarities between MHC molecules. It is probable that allo reactivity is mediated by self MHC restricted antigen specific T cells since many T cell clones can mediate both types of T cell recognition [105-108]. The fine line between self MHC and altered self MHC or allo MHC calls for some means of eliminating T cell clones with fortuitously high affinity for self MHC molecules.

Although it is possible that self MHC tolerance is controlled in part by T suppressor cells [109,110], extensive evidence has been obtained in support of a clonal deletion mechanism for the removal of unwanted T cell specificities.

The T cell receptor and negative thymic selection

Kappler et al.[111] were the first to demonstrate that the natural correlation between a TcR specificity directed by the Vβ17a gene and a self determinant, namely self MHC Class II I-E molecules, results in the deletion of mature Vβ17a bearing T cells in mouse strains expressing the I-E MHC gene products. These authors also found that immature thymocytes did express the Vβ17a gene product suggesting that tolerance induction occurs in the thymus and acts on immature thymocytes. Other natural correlations between specific TcR genes and self determinants that have been identified also result in similar clonal deletions [112,113]. Kappler et al.[114] find that T cells expressing the TcR Vβ8.1 gene product are deleted in $H\text{-}2^k$ or $H\text{-}2^b$ but not in $H\text{-}2^q$ mice bearing the minor lymhocyte stimulatory (Mls) antigen, $Mls\text{-}1^a$, while Pullen et al.[115] observe the deletion of T cells expressing the TcR Vβ3 gene product in $H\text{-}2^k$ mice bearing the Mls antigen, $Mls\text{-}2^a$. Similarly, MacDonald et al.[92] observe the deletion of T cells expressing the TcR Vβ6 gene product in $H\text{-}2^k$ or $H\text{-}2^d$ mice bearing the $Mls\text{-}1^a$ antigen and show that this deletion occurs only when appropriate MHC Class II molecules are expressed [93].

Teh et al.[96] studied T cell clonal deletion in transgenic mice expressing an $H\text{-}2^b$ derived CTL TcR specific for the male antigen H-Y in the context of Class I $H\text{-}2D^b$ MHC molecules. They showed that deletion of T cells expressing this transgene occurs only in males but not in females bearing the appropriate $H\text{-}2^b$ MHC molecules. In addition, the deletion of transgene bearing T cells in males affects both CD4$^-$CD8$^+$ as well as CD4$^+$CD8$^-$ cells implying that deletion occurred at the CD4$^+$CD8$^+$ thymocyte stage. Furthermore, Kisielow et al.[116] show that only T cells expressing relatively high cell surface density of CD8, including CD4$^+$CD8$^+$ thymocytes, were subject to deletion. Another transgene system makes use of an H-2^b derived CTL TcR specific for the L^d Class I MHC antigen. Sha et al.[95] demonstrate that the transgenic H-2$^{b/d}$ but not H-2^b haplotype deletes functional T cells expressing the L^d specific CTL TcR transgene. Deletion also appears to occur at or before the CD4$^+$CD8$^+$ thymocyte stage since the transgenic receptor was predominantly detected on CD4$^-$CD8$^-$ cells in the H-2$^{b/d}$ animals.

The consequences of deleting T cells bearing various self reactive TcR's can lead to non-responsiveness to specific foreign antigens as recently described by Vidovic & Matzinger [117] for the synthetic antigen (GT). Alternatively, the immune system will utilize other TcR's to compensate for the deleted ones. In the cytochrome c system, most Ia (Eα^k Eβ^k)-restricted T cells from B10.A mice express a product of the Vα11 gene family in association with a Vβ3 gene encoded protein [118]. Fry & Matis [119] observe that several mouse strains fail to express any Vβ3 due to tolerance induced by Mls-2^a self antigens which are specifically recognized in the context of H-2^k Class II MHC molecules by Vβ3 bearing T cells [120]. Although the strains that deleted Vβ3 bearing T cells used other Vβ TcR genes in their cytochrome c specific responses, the fine antigen specificity between cytochrome c specific T cells from Vβ3$^+$ and Vβ3$^-$ strains were found to be different. Therefore the antigen specific T cell repertoire is affected by the deletion of self reactive T cells.

Cell types in thymus controlling negative thymic selection

There is an emerging consensus that epithelial cells play little or no part in tolerance induction while intrathymic bone marrow derived cells, probably macrophage / DC, play a decisive role in tolerance induction. Zinkernagel et al.[79] observed that in contrast to untreated neonatal thymuses, nude mice grafted with irradiated MHC different thymuses failed to induce mixed lymphocyte reaction (MLR) and CTL tolerance to allograft antigens. Likewise depleting thymuses of macrophage / DC in vitro by culture with deoxyguanosine (dGuo) results in thymus grafts incapable of inducing tolerance to thymic allo antigens when used to reconstitute T cell function in nude mice [121]. Also, stem cells differentiating in vitro in dGuo treated thymuses do not develop tolerance to MHC alloantigens of the thymus [122].

In contrast, the presence of macrophage / DC in untreated thymuses invariably results in the development of T cells tolerant to the graft allo-antigens [121,123]. In irradiation chimeras donor derived macrophage / DC rapidly appear in the thymus [101,124] and T cells differentiating in such MHC different thymuses always show tolerance to autologous MHC determinants [125,126]. Finally, supplementing lymphoid cell-depleted thymuses with lymphohemopoietic cells has been shown to restore tolerogeneicity of the thymus graft [122].

Determinant selection limits the expressed T cell repertoire : Association of Class II MHC molecules and antigen

Since T cells do not respond to soluble antigens but rather recognize antigen only in the context of self MHC molecules on the surface of antigen presenting cells (APC), some foreign antigenic epitopes may not have the ability to bind to the MHC molecules and will thus not be recognized by the expressed

T cell repertoire. This determinat selection mechanism, originally proposed by Rosenthal et al.[127] to explain Ir-gene controlled nonresponsiveness in guinea pigs, has been supported by experiments showing that peptides can compete for binding to Ia molecules on the surface of antigen presenting cells. Thus, antigen specific T cell proliferation can be specifically inhibited by structurally related non stimulatory peptides at the level of antigen presentation[128]. However, direct evidence that antigen and Ia molecules interact has only recently been obtained. Babbit et al.[129] have shown that the hen egg lysozyme (HEL) fragment 46-61, which is immunogenic in H-2^k but not in H-2^d mice, associates specifically with detergent solubilized I-A^k but not I-A^d molecules in equilibrium dialysis experiments. Similar results were also obtained by Buus et al.[130] demonstrating that the immunogenic ovalbumin peptide 323-339 bound specifically to I-A^d, its usual restricting element, but not to irrelevant Ia molecules. Since antigen and Ia molecules must interact for T cell stimulation to occur, the generation of appropriate antigenic peptides as a result of antigen processing by antigen presenting cells must be a critical step in this process.

Influence of antigen processing on determinant selection

The handling of antigen by macrophage antigen presenting cells(APC) has been studied extensively by Unanue [4]. It has been shown that antigen processing involves a temperature sensitive and energy requiring step which can be blocked by fixation of the APC by paraformaldehyde [131]. This antigen processing event can involve merely denaturation [132,133] although fragmentation of the antigen within the acidic environment of a lysosome is usually required [134] and can be profoundly inhibited by lysosomotropic agents such as chloroquine and ammonium chloride which raise the intralysosomal pH level [135]. Shimonkevitz et al.[136,137] were the first to demonstrate that antigen fragmentation was a necessary and sufficient step for the activation of two Class II restricted ovalbumin specific T cell hybridomas which could not respond to native or denatured ovalbumin on fixed APC but could respond to enzymatic or synthetic fragments of ovalbumin on these fixed APC.

Other lines of evidence that processing played a major role in T cell repertoire expression came from experiments comparing peptides versus native molecules as immunogens. Brett et al.[138] demonstrated that equine myoglobin does not elicit T cell responses in $H\text{-}2^k$ mice to the fragment 102-108 although this fragment is perfectly immunogenic in $H\text{-}2^k$ mice. Similar observations were made by Gammon et al. [139] using hen egg lysozyme (HEL) and its fragment 74-96 in B10.A mice. In vivo processing of native molecules thus appears to be inadequate in the generation of some peptide fragments possibly as a result of inadequate degradation or alternativelly as a result of excessive degradation. Antigen processing events also appear to influence the choice of two distinct T cell determinants within a 23 amino acid region 74-96 of HEL depending on its structural context [140]. Thus, native HEL and denatured HEL molecules elicit phenotypically different T cells with specificity for the HEL fragment 74-96. In addition, differential processing of species variant lysozymes appears to result in the heteroclicity of B6 clones for ring-necked pheasant lysozyme (REL) in preference to HEL [141]. HEL specific B6 clones respond better to native REL than native HEL but respond equally well to REL and HEL fragments containing the necessary epitopes for recognition.

Several investigators have suggested that MHC molecules themselves play a role in antigen processing. Gammon et al.[139] have proposed that competition may occur between multiple MHC binding sites present on the same processed fragment. Thus, if a partially unfolded fragment first binds MHC by one of its MHC binding sites which is already exposed, further processing may stop and the other MHC binding sites are never exposed. A similar hypothesis, namely determinant protection, has been forwarded by Werdelin [142]. The author suggests that Ia molecules may bind small fragments of protein antigens and protect them against proteolytic destruction within antigen presenting cells. Support for these ideas has recently been obtained by Donermeyer et al.[143] who demonstrate that the HEL fragment 40-73 becomes resistant to proteolytic degradation by chymotrypsin only after it is allowed to associate with I-A^k molecules on the surface of fixed macrophage APC. These studies are also consistent with the observation that the recently crystalized membrane distal domains α_1 and α_2 of the human Class I MHC molecule (HLA-A2) were found to contain an unidentified stucture, possibly a processed peptide, in the cleft which is formed by two parallel helices lying on top of a platform consisting of 8 anti-parallel β-strands [144,145].

While processing of small peptides is not necessary for presentation, Fox et al,[146] demonstrate that processing of a minimal peptide can alter its interaction with MHC molecules. The choice of peptide presented by any given MHC molecule may also be the result of competition between available MHC molecules. Thus, even if a peptide is generated during antigen processing it may preferentially associate with one particular MHC molecule. For instance, when the HEL fragment 13-35 is generated in I-A^d/I-E^d bearing mice it associates exclusively with the I-E^d molecule. However, when that same fragment is generated in I-A^d/I-E^o bearing mice it associates with the I-A^d molecule [139].

Finally, antigen processing events may destroy or reveal certain structural features important in determining epitope immunogenicity. A statistical analysis of well defined immunogenic T cell epitopes revealed that almost all these epitopes are representative of amphipathic alpha-helices which have their hydrophobic residues and their hydrophilic residues segragated in space on opposite sides of the alpha-helix [147]. It is not presently clear whether such helices are preferentially spared proteolytic degradation or if they bind more effectively to MHC molecules or APC cell surfaces thereby giving them a selective advantage over other T cell epitopes.

Although we have most extensive information regarding antigen processing for presentation to Class II restricted T lymphocytes, recent evidence suggests that Class I restricted T lymphocytes also recognize degraded or "processed" forms of protein antigens [148]. The original assumption that Class I restricted CTL cells recognize intact foreign glycoproteins ie. virus specific antigens inserted alongside MHC molecules in the membranes of target cells [149-151] has been challanged by experiments demonstrating that fragmented forms of specific viral polypeptides [152-154] as well as expressed products of truncated viral genes [155-157] can stimulate Class I restricted CTL responses. In addition, several investigators have sensitized CTL target cells using synthetic oligopeptides corresponding to specific portions of viral polypeptides [158-160].

However, the epitopes recognized by Class I restricted T cells do not appear to be generated by the pH dependent pathway, involving antigen uptake, intralysosomal degradation and re-expression of antigen on the cell surface of APC's, used to present soluble and particulate antigens to Class II restricted T cells [4]. Exposing CTL target cells to purified undenatured protein antigens ie. influenza nucleoprotein [161], chicken ovalbumin [162], or hemagglutinin [157,163] does not sensitize them for lysis by Class I restricted CTL cells. Only target cells that synthesize these proteins are lysed by such CTL cells. The epitopes recognized by Class I restricted T cells thus appear to be the degradation products of proteins which have been subjected to a cytoplasmic degradation system. Proteins which are exposed to the cytoplasm include de novo synthesized proteins expressed in transfected [155,162,164,165] or virus infected [156,157,166-168] cells. Exogenous nonfragmented proteins can be presented to Class I restricted T cells only if they enter target cells through endosomal or pinosomal membranes and are exposed to the cytosol of those target cells [162,169]. Although the nature of the proteolytic system involved in the presentation of cytoplasmic proteins to Class I restricted T cells remains unknown, it is unlike the antigen presentation pathway for Class II restricted T cells in that it is not inhibited by the lysosomotropic agent chloroquine while it is inhibited by the protein synthesis inhibitor emetine [159,163].

Finally, the experiments of Morrison et al. [163] demonstrating that souble hemagglutinin (HA) can sensitize target cells for lysis by Class II but not Class I restricted CTL cells while target cells which synthesize HA are lysed by Class I but not Class II restricted CTL cells suggest that Class II and Class I MHC molecules may actually be physically segragated with the degradation products of exogenous and de novo synthesized protein antigens, respectively [170-173].

Immunodominance and the T cell repertoire

The T cell response to many foreign antigens has been found to be focused on a limited number of epitopes. Such focusing of the immune response not only occurs with species homologous protein antigens such as myoglobin [174,175], insulin [176], cytochrome c [177,178] and lysozyme [179,180] which only bear a limited number of nonself sites, but also with non eukaryotic protein antigens which are unrelated to self proteins such as influenza hemeagglutinin

[181], beta-galactosidase [182] and staphylococcal nuclease [183]. This focusing is predominantly influenced by the particular MHC genes expressed. It has been shown in the lysozyme antigen system quite clearly that there are no absolutely immunodominant regions specific for the mouse species but that each MHC haplotype determines its own focus of reactivity. Mice of H-2^k [179], H-2^b [184], H-2^d [185] and H-2^q [186] haplotypes focus on different regions of the hen egg lysozyme(HEL) antigen. Similar observations have been made for insulin [187,188] and cytochrome c [189,190] presented in the context of various Class II MHC molecules. The influence of MHC molecules on immunodominance may reflect the ability of various processed fragments to bind to particular Class II MHC molecules. In support of this idea, peptides representing immunodominant epitopes of antigens such as ovalbumin [130] and HEL [129] have been shown to bind specifically to soluble Ia molecules representing their usual restricting elements but not to irrelevant Ia molecules. High affinity interactions with MHC molecules would be consistent with observations that immunodominant epitopes are generally highly immunogenic and elicit strong T cell responses.

An interesting feature of T cell responses directed at immunodominant epitopes is their heterogeneity. T cell clones and T cell hybridomas specific for various immunodominant epitopes have been analyzed for fine antigen specificity using proteolytic fragments as well as related synthetic peptides of various protein antigens. Haplotype variant spleen cells have also been used for the presentation of antigens or in the absence of antigen to determine allocrossreactivity patterns. In cytochrome c, at least 5 phenotypically distinct groups of T cell clones respond to the immunodominant epitope 81-103 in the context of I-E^k MHC molecules [191]. Similarly, sperm whale myoglobin (SWM) contains several immunodominant epitopes which elicit heterogeneous T cell responses [192,193]. At least 3 different I-E^d restricted and 3 different I-A^d restricted T cell clones respond to the 11 amino acid long SWM epitope 110-121 and the 12 amino acid long SWM epitope 106-118, respectively [175]. Hen egg lysozyme fragments 46-61 and 34-45 (12,16) or 74-86 and 81-96 (13,14), have also been shown to elicit heterogeneous T cell responses. Even the hapten p-Azobenzenearsonate has been shown to elicit at least 3 different T cell specificities in A/J mice [194]. Phenotypic heterogeneity of the T cell populations directed at immunodominant epitopes would therefore appear to be the rule rather than the exception.

The use of dominant TcR Vα and/or Vβ genes has been observed in several of these antigen systems and with some possible exceptions [195,196] does not appear to limit the phenotypic potential of their expressed T cell repertoires. The T cell responses elicited by cytochrome c, p-Azobenzenearsonate and SWM are quite heterogeneous even though Vα11 is used by the majority of cytochrome c specific T cells [118,197,198], Vα3 by most arsonate specific T cells [199] and Vβ8 by almost all I-E^d but not I-A^d restricted SWM specific T cells [175]. The use of dominant V region genes may therefore reflect the preferential selection of some TcR chains in the thymus based on general epitope specificities which express many different fine epitope specificities in combination with different V region gene partner chains.

Immunodominance may also be a reflection of the absence of T cells in the expressed T cell repertoire as a result of negative selection during thymic development because of a homology with self protein antigens. Deletion of autoreactive T cell clones can lead to complete nonresponsiveness to a particular antigen as has recently been demonstrated by Vidovic & Matzinger[117] for the synthetic antigen (GT). Alternatively, it may lead to the selection of T cells with altered phenotypes as was observed in the cytochrome c system when one of the dominant TcR Vβ genes, Vβ3, was deleted and alternative TcR Vβ genes were selected instead [119]. The antigen specific T cell repertoire may therefore be subtly affected by the deletion of self reactive T cells.

At least in some instances immunodominance is also determined by suppressor machanisms. Epitopes of several antigens including lysozyme [200,201], poly-(Glu,Ala,Tyr) [202], β-galactosidase [203] and insulin [204] have been shown to induce suppressor T cells in selected strains of mice. Suppressive regulatory influences are therefore also strongly influenced by the particular MHC genes expressed.

Finally, immunodominance has also been shown to be affected by antigen processing and the structural context within which epitopes are presented to the immune system so as to encourage or hinder the antigen processing events as discussed earlier [139,146,205,206].

PROJECT AND RATIONALE

Several antigenic systems have been used to study the nature of the expressed T cell repertoire. These studies can be separated into two general categories. First, thymic selection of the T cell repertoire including positive and negative selection of self MHC restricted T cells and second, post thymic selection of the T cell repertoire including antigen processing and determinant selection.

A. Synthetic peptide antigens such as the random polymers (T,G)-A-L and GAT have predominantly been used to study Ir gene controlled immune nonresponsiveness. These studies focused on thymic selection and several investigators have demonstrated that a responder thymic environment can select for Class II restricted T cells expressing the responder phenotype even if the T cells themselves are phenotypic nonresponders [82,87,88]. This suggests that there are no limitations in the ability of nonresponder T cells to generate a full range of T cell specificities and that it is the environment in which the T cells mature which dictates the extent and specificity of the expressed T cell repertoire. However, an issue which has not been addressed by these experiments, since the T cell responses to the synthetic random polymer antigens have not been clearly defined at the clonal level, is whether responder T cells and nonresponder T cells educated in a responder thymic environment generate phenotypically similar or dissimilar T cell clones in response to a given Ir-gene controlled antigen. In otherwords, is there a contribution of the T cell genotype to the selection of the expressed T cell repertoire.

Our objective was to answer this question using the well defined Ir-gene controlled antigen system Poly-18. Poly-18 is a synthetic polymer of the 18 amino acid sequence, $EYK(EYA)_5$, with an average MW of 11000. This polypeptide also has a well defined secondary structure and forms a regular alpha helix wherein the lysine residues are separated spatially by 27 angstroms and line up on one face of the alpha helix [207,208]. Responsiveness to Poly-18 is under Ir gene control. Mice of the $H\text{-}2^d$ haplotype ie. BALB/c and DBA/2J are responders and elicit Poly-18 specific antibodies, DTH and proliferating T cells while mice of the $H\text{-}2^b$ haplotype ie. C57BL/6, C57BL/10 and C3H/SwSn or $H\text{-}2^k$ haplotype ie. CBA/J, CBA/CaJ, B10.Br and C3H/HeJ are Poly-18 nonresponders. Evidence of T cell mediated suppression was not found in Poly-18 nonresponder mouse strains [208]. In the responder BALB/c($H\text{-}2^d$) mice, Poly-18 elicits two major T cell clonotypes, Type A and Type B. Type A T cell hybridomas specifically recognize the lysine containing epitope $EYK(EYA)_4$ while Type B T cell hybridomas respond to the lysine free epitope $(EYA)_5$ [209].

The use of synthetic fragments representing the dominant epitopes of the Poly-18 antigen enabled us to analyse the full range of H-2^d restricted T cell specificities generated by T cells of the responder H-2^d, H-2^{bxd} and nonresponder H-2^b genotypes which were educated in H-2^d, H-2^{bxd} and H-2^{bxd} responder thymic environments, respectively (Chapter 3).

B. Experiments examining antigen processing requirements and the effects of determinant selection on the expressed T cell repertoire have been studied most extensively using species homologous protein antigens such as cytochrome c, lysozyme or insulin or related proteins such as ovalbumin and myoglobin as well as several non eukaryotic protein antigens. The recognition of all these antigens by the immune system has been found to be focused onto a small number of epitopes which are capable of eliciting vigorous and often heterogeneous T cell responses. However, an extensive analysis of T cell clonotypes directed at a well defined synthetic polymer such as Poly-18, which has a simple repeating amino acid sequence and a well defined secondary structure, has not been done.

Our first objective was to determine whether immune focusing occurs in response to a repeating antigen such as Poly-18, given that all of its epitopes have extensive amino acid sequence overlap. We have previously determined the minimum Poly-18 peptide sequences required for the priming and triggering of Poly-18 specific Ab, DTH and T cell proliferative responses in BALB/c(H-2^d) mice. The 9 amino acid peptide $EYK(EYA)_2$ is sufficient to elicit Ab and proliferative T cells in vivo but only the 12 amino acid peptide $EYK(EYA)_3$ or longer peptides can elicit T cell prolifera[illegible] responses in vitro. Also, the 12 amino acid peptide $EYK(EYA)_3$ is sufficient to induce DTH T cells but only the 18 amino acid peptide $EYK(EYA)_5$ or Poly-18 can subsequently elicit the DTH response [210]. We have also determined that the minimum Poly-18 peptide sequences required for the triggering of Poly-18 specific T cell hybridomas are 15 amino acids long and that neither Poly-18 nor the Poly-18 related minimum peptides require antigen processing prior to presentation to the T cell hybridomas [211].

We therefore used 12 and 15 amino acid long synthetic Poly-18 fragments, representing all the possible sequential Poly-18 epitopes, to stimulate Poly-18 specific proliferative T cells and T cell hybridomas in vitro and to elicit Poly-18 specific T cells in vivo in order to identify which of the fragments represent immunodominant epitopes of the Poly-18 antigen (Chapter 2).

Our second objective was to determine whether an epitope which can be defined as immunodominant ie. $(EYA)_5$ is capable of eliciting a heterogeneous T cell response, given that it has a relatively simple amino acid sequence. We analyzed the diversity of the I-A^d restricted, Poly-18 specific, T cell response directed at the immunodominant Poly-18 epitope, $(EYA)_5$, by performing a fine antigen specificity analysis on a series of I-A^d restricted, $(EYA)_5$ specific, T cell hybridomas using a panel of 15 amino acid long synthetic Poly-18 related peptides (Chapter 4).

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CHAPTER 2

Hierarchy of antigenic determinants in the synthetic polypeptide antigen Poly-18

(Paper I)

Introduction

The recognition of foreign antigens by the immune system has been found to be focused onto a small number of epitopes which are capable of eliciting vigorous and often heterogeneous T cell responses. In species homologous protein antigens, such as cytochrome c [1,2], insulin [3] or lysozyme [4,5] only a few nonself sites are actually present. However, the focusing of the immune response onto limited regions of foreign molecules has also been demonstrated for non eukaryotic proteins which are phylogenetically distant from the self proteins of the mouse, such as influenza hemagglutinin [6], beta-galactosidase [7] and staphylococcal nuclease [8]. Many factors contribute to this phenomenon of epitope immunodominance. One major factor influencing the selection of the expressed T cell repertoire is the major histocompatibility complex (MHC). The selection of immunodominant epitopes on antigens such as hen egg lysozyme [9], sperm whale myoglobin [10], staphylococcal nuclease [11,12] and insulin [13] has been shown to depend on the MHC molecules present in different strains of mice. The influence of MHC on the expressed T cell repertoire occurs intrathymically at the time of positive and negative selection of T cells which are both self MHC restricted and self tolerant [14-17] as well as extrathymically at the time of antigen presentation. Since T cells do not respond to soluble antigen but rather recognize antigen only in association with self MHC molecules on the surface of antigen presenting cells (APC) [18], some epitopes may not have the ability to bind to self Class II MHC (Ia) molecules and will thus not be presented to available T cells [19,20]. Another major factor influencing the selection of the expressed T cell repertoire is antigen processing. Since most large protein antigens need to be degraded or processed by APC [21] prior to association with self MHC molecules for presentation to T cells, the antigen processing event may selectively reveal by denaturation [22,23] or destroy by fragmentation particular T cell epitopes. In addition, the MHC may also be involved in the antigen processing event. It has been suggested that MHC molecules may bind to partially degraded protein molecules and thereby protect selected epitopes [24,25] as well as prevent other epitopes from binding to the already occupied MHC binding site [26]. As a result of all these influences on the expressed T cell repertoire, the immune response appears to be hierarchical with some epitopes being recognized more favourably than others.

We analysed the hierarchy of antigenic determinants in the synthetic polypeptide antigen Poly-18, PolyEYK$(EYA)_5$ [27]. This polypeptide has a well defined sequence and forms a regular alpha-helix. Responsiveness to Poly-18 is under Ir gene control. Mice of the H-2^d haplotype are responders and elicit Poly-18 specific antibodies, DTH and proliferating T cells while mice of the H-2^b or H-2^k haplotypes are Poly-18 nonresponders [28]. We have also shown that the 9 and 12 amino acid peptides ie. EYK$(EYA)_2$ and EYK$(EYA)_3$ are sufficiently long to elicit T cell responses in vivo but only peptides of 12 amino acids or longer can elicit T cell responses in vitro [29]. Since Poly-18 is a repeating antigen we have been able to synthesize a series of peptides representing all the possible 12 and 15 amino acid long sequential overlapping epitopes of the Poly-18 antigen. Poly-18 and its fragments do not need to be processed prior to being presented to Poly-18 specific T cells [30]. We therefore used the synthetic peptides representing the sequential Poly-18 epitopes to identify immunodominant epitopes of the Poly-18 antigen using the criteria of epitope immunogenicity, the ability of the epitope to elicit an immune response in vivo, and epitope antigenicity, the ability of the epitope to elicit a secondary immune response in vitro. Our analysis indicates that Poly-18 has 2 immunodominant epitopes, $(EYA)_4$ [or $(EYA)_5$] and $EYK(EYA)_3$ [or $EYK(EYA)_4$] and that immunodominance is strictly correlated with epitope immunogenicity as well as epitope antigenicity. The epitope $(EYA)_3EYK$ appears to be subdominant since it can elicit Poly-18 specific T cells when it is used as the immunogen but does not appear to be part of the Poly-18 repertoire when Poly-18 is used as the immunogen. Poly-18 elicits two T cell clonotypes, Type A and Type B, which are directed at the two immunodominant Poly-18 epitopes [31]. Type A T cell hybridomas respond to the lysine containing epitope $EYK(EYA)_4$ while Type B T cell hybridomas respond to the lysine free epitope $(EYA)_5$ as well as the lysine containing epitope $EYK(EYA)_4$. Our analysis indicates that the Type B clonotype is very heterogenous and much larger than the Type A clonotype and that this is not related to the availability of the lysine containing epitope $EYK(EYA)_4$. Our analysis also indicates that peptides representing the two immunodominant Poly-18 epitopes elicit a broader range of clones than the whole Poly-18 molecule.

Materials and Methods

Mice- BALB/c(H-2^d) mice were bred at the Ellerslie Animal Farm of the University of Alberta, Canada. Female mice were used at 2-6 mo of age.

Peptides- Poly-18 and the various related peptides were prepared by the Merrifield solid phase peptide synthesis technique [32] as described previously [27,33]. For functional assays, peptides were dissolved in saline, pH adjusted to 7.2 with 0.1N NaOH, sterilized by filtration through a 0.22 Um filter and stored at -20'C. Amino acid abbreviations: K; lysine, E; glutamic acid, Y; tyrosine, A;alanine.

Lymph node T cell proliferation assay- BALB/c mice(2-5 per group) were primed in both hind footpads with 30μg of antigen in complete freunds adjuvant(CFA). 7-9 days later popliteal lymph nodes were removed and a single cell suspension was prepared. Cultures were set up in duplicate or triplicate at 6.5×10^5 viable lymph node cells and 20μM antigen in 250 μl RPMI 1640 (Gibco) containing 10% v/v heat inactivated Human serum, 5×10^{-5} M 2-mercaptoethanol, 10 mM Hepes, 2 mM glutamine and penicillin-streptomycin. Cultures were pulsed 4 days later with 0.7 μCi of [methyl-^{3}H]-thymidine for 4-6 hours prior to harvesting.

T cell lines- BALB/c mice were primed as above with either Poly-18, EYK$(EYA)_3$ or $(EYA)_5$. 7-9 days later popliteal lymph node T cells were nylon wool separated and $2\text{-}4\times10^5$ T cells/ml were cultured with 3×10^6 irradiated(4000 R) BALB/c spleen cells/ml and 2 μM priming antigen in RPMI 1640 (Gibco) containing 10% fetal bovine serum(FCS), 5×10^{-5} M 2-mercaptoethanol, 10 mM Hepes, 2 mM glutamine and penicillin-streptomycin. T cell lines were carried for 2-3 weeks in vitro prior to fusing with BW5147 thymoma cells.

T cell fusions- T cell fusions were performed as previously described using a standard PEG fusion protocol [31]. For fusing Poly-18 or EYK(EYA)$_3$ specific T cell lines the standard AKR thymoma BW5147 was used, and for fusing the (EYA)$_5$ specific T cell line the BW5147α–β- thymoma, a derivative of BW5147 which does not express the alpha and beta chains of the T cell receptor (kindly made available to us by Drs. P. Marrack & J. Kappler, Denver) was used. After fusion, cells were plated in 24 well linbro plates at 2x10^5 BW5147 cells/2ml/well. T cell hybridomas were initially grown in Hypoxanthine-Thymidine medium for two weeks and eventually adapted to drug free medium containing only RPMI 1640 and 10% FCS. Hybridomas were then tested for IL-2 release in the presence of various antigens and irradiated (4000 R) spleen cells as APC or subcloned by limiting dilution prior to testing.

T cell hybridoma antigen specificity analysis- The antigen specificity of T cell hybridomas from individual linbro wells (Fig 2.2) or recloned T cell hybridomas (Table 2.3 and 2.4) was tested by incubating T cell hybridoma cells (10^5) with (10^6) BALB/c H-2^d irradiated spleen cells and 10μM antigen in 300μl RPMI 1640 and 10% FCS for 24 hours. The supernatants were diluted at 1:2 in 200 μl and tested for the presence of IL-2 by their ability to support the growth of 10^4 IL-2-dependent CTLL cells, as measured by ^{3}H-thymidine incorporation.

Results

Sequential epitopes of Poly-18 .

Poly-18 is a synthetic alpha helical polypeptide which is a polymer of the 18 amino acid sequence $EYK(EYA)_5$. We synthesized a series of 12 and 15 amino acid long peptides which represent all the possible sequential epitopes of the Poly-18 antigen. (Table 2.1) Since Poly-18 is a repeating antigen, all the possible sequential epitopes have considerable amino acid sequence overlap. The main distinguishing feature of the Poly-18 epitopes is the position of the lysine residue within the 12 and 15 amino acid long peptides. Some of the epitopes do not contain any lysine residues ie. $(EYA)_4$ and $(EYA)_5$. Some of the epitopes contain lysine residues near the N-terminus ie. $EYK(EYA)_3$ and $EYK(EYA)_4$ while others contain C-terminal lysine residues ie. $(EYA)_3EYK$ and $(EYA)_4EYK$. The epitope $EYAEYK(EYA)_3$ has a lysine residue close to the N-terminus while $(EYA)_3EYKEYA$ has a lysine residue close to the C-terminus. Epitopes which have a lysine residue as close to the centre of the epitope as possible are $EYAEYK(EYA)_2$, $(EYA)_2EYKEYA$ and $(EYA)_2EYK(EYA)_2$.

We synthesized peptides of 12 as well as 15 amino acid lengths since the 12 amino acid long Poly-18 derived peptides can be used for in vivo T cell priming as well as stimulation of in vitro T cell proliferative responses [29]. Unlike normal T cells, T cell hybridomas generated by fusing Poly-18 specific T cells with the thymoma BW5147 require 15 amino acid long Poly-18 derived peptides for optimal activation [30,31]. Furthermore, our analyses of Poly-18 specific T cell hybridomas indicate that the 15 amino acid long peptides do not require antigen processing and can be presented to the T cell hybridomas on glutaraldehyde fixed APC [30].

Immunodominant epitopes of Poly-18.

Our previous studies suggested that the peptides containing lysine residues near the N-terminus i.e. EYK(EYA)$_2$, EYK(EYA)$_3$ and EYK(EYA)$_5$ elicit Poly-18 specific T cell responses in BALB/c mice [29]. Using the more extensive panel of 12 and 15 amino acid long Poly-18 derived peptides (Table 2.1) we identified two Poly-18 immunodominant epitopes. The Poly-18 specific T cell response in BALB/c mice is directed exclusively to the two epitopes represented by the 12 amino acid lysine containing peptide EYK(EYA)$_3$ and the lysine free peptide (EYA)$_4$. (Fig 2.1a) There appear to be no T cells generated to the 12 amino acid peptides containing centrally located lysine residues ie. (EYA)$_2$EYKEYA and EYAEYK(EYA)$_2$ or C-terminal lysine residues ie. (EYA)$_3$EYK in these mice. The Poly-18 specific T cells from BALB/c mice also respond to the 15 amino acid peptides EYK(EYA)$_4$, EYAEYK(EYA)$_3$, (EYA)$_5$ and (EYA)$_4$EYK. (Fig 2.1b) This pattern of response is entirely consistent with epitope overlap between the 12 and 15 amino acid long peptides. (Table 2.1) The 15 amino acid long peptides EYK(EYA)$_4$ and EYAEYK(EYA)$_3$ have amino acid sequence overlap with the lysine containing peptide EYK(EYA)$_3$ while the 15 amino acid long peptides (EYA)$_5$ and (EYA)$_4$EYK have amino acid sequence overlap with the lysine free peptide (EYA)$_4$. No Poly-18 specific T cells response is detected against the peptides containing centrally located lysine residues ie. (EYA)$_2$EYK(EYA)$_2$ or a C-terminal lysine residue ie. (EYA)$_3$EYKEYA.

Immunogenic and antigenic potential of Poly-18 derived peptides.

We analysed the ability of the various Poly-18 derived peptides to elicit peptide and/or Poly-18 specific T cells in vivo and to stimulate the elicited T cells to proliferate in vitro. (Table 2.2) Peptides $EYK(EYA)_3$ and $EYK(EYA)_4$, representing N-terminal lysine containing Poly-18 epitopes, are strongly immunogenic and elicit Poly-18 specific as well as peptide specific T cells in vivo. These two peptides are also strongly antigenic and elicit peptide specific T cell proliferative responses in vitro. Similarly, peptides $(EYA)_4$ and $(EYA)_5$, representing lysine free Poly-18 epitopes, are strongly immunogenic and elicit Poly-18 specific as well as peptide specific T cells in vivo. These lysine free peptides are also strongly antigenic and elicit peptide specific T cell proliferative responses in vitro. Alternatively, peptides $(EYA)_3EYK$ and $(EYA)_3EYKEYA$, representing Poly-18 epitopes containing lysine residues near the C-terminus are only weakly immunogenic and elicit relatively fewer Poly-18 specific T cells in vivo than the lysine free or N-terminal lysine containing peptides. Peptides $(EYA)_3EYK$ and $(EYA)_3EYKEYA$ are also very poorly antigenic and only peptide $(EYA)_3EYK$ is able to marginally stimulate $(EYA)_3EYK$ specific T cells to proliferate in vitro. Peptides containing centrally located lysine residues such as $(EYA)_2EYKEYA$, $EYAEYK(EYA)_2$ and $(EYA)_2EYK(EYA)_2$ appear to be completely non immunogenic and do not elicit any detectable Poly-18 specific or peptide specific T cell responses. Strong immunogenicity as well as strong antigenicity therefore appear to be characteristic only of the peptides which represent immunodominant epitopes of Poly-18.

T cell clonotypes directed at immunodominant epitopes of Poly-18 : Analysis with T cell hybridomas.

We have previously identified two major Poly-18 specific T cell clonotypes, Type A and Type B, elicited by Poly-18 in BALB/c (H-2^d) mice [31]. Type A clones are absolutely dependent on the presence of lysine in position 3 and respond to the Poly-18 derived peptide EYK$(EYA)_4$ while Type B clones are lysine independent and respond to the Poly-18 derived peptide $(EYA)_5$. Although Type B clones are lysine independent, they do respond to peptides containing lysine residues at position 3 ie. EYK$(EYA)_4$ and to a lesser extent at position 15 ie. $(EYA)_4$EYK. (Table 2.3) However, only a subpopulation of Type B clones respond to the peptide EYK$(EYA)_3$EYK (Abbr. K3K) which contains lysine residues at both positions 3 and 15 while all Type A clones respond to this peptide. Our analysis indicates that Type B clones actually represent a whole spectrum of clonotypes some of which do not respond to the K3K peptide at all, some of which respond to the K3K peptide only at very high antigen concentrations ie. 100μM and some of which respond to the K3K peptide even at low antigen concentrations ie. 10μM. (Table 2.4) T cell clones which do not respond to the low 10μM concentrations of K3K are considered to be weakly K3K reactive while clones which can respond to the low 10μM concentrations of K3K are considered to be strongly K3K reactive. The strongly K3K reactive clones therefore consist of all Type A clones and a subpopulation of Type B clones. We can thus represent the Poly-18 specific T cell repertoire as a spectrum of clones ranging from no K3K reactivity to high K3K reactivity and simultaneously ranging from high $(EYA)_5$ reactivity to no $(EYA)_5$ reactivity. (Table 2.4)

Relative frequency of $(EYA)_5$ specific and K3K specificT cell clones in the Poly-18 specific T cell repertoire of BALB/c mice : Analysis with T cell hybridomas.

We analysed the relative frequency of $(EYA)_5$ specific and K3K specific T cell clones present in a Poly-18 specific T cell population. The $(EYA)_5$ specific T cell clones represent only Type B clones while the K3K specific T cell clones represent all Type A clones as well as the strongly K3K reactive subpopulation of Type B clones. Poly-18 specific T cell hybridomas were generated by fusing Poly-18 specific T cells with BW5147 thymoma cells. T cell hybridomas from primary fusion wells were then assessed for their ability to respond to the lysine free peptide $(EYA)_5$ or the lysine containing peptide K3K at an antigen concentration of 10μM. The results in Fig. 2.2a indicate that the ratio of $(EYA)_5$ specific to K3K specific T cell clones is approximatelly 2:1 in the Poly-18 specific T cell population. Eventhough the K3K specific T cell clones do not represent only Type A clones but also the strongly K3K reactive subpopulation of Type B clones, they occur only half as frequently as the $(EYA)_5$ specific T cell clones. This indicates that Type B clones are much more frequent than the Type A clones in the Poly-18 specific T cell repertoire.

Relative frequency of $(EYA)_5$ specific and K3K specific T cell clones in the $EYK(EYA)_3$ and $(EYA)_5$ specific T cell repertoires of BALB/c mice : Analysis with T cell hybridomas.

In order to establish whether structural features of the Poly-18 antigen were contributing to the frequency distribution of the $(EYA)_5$ and K3K specific T cell clones in the Poly-18 specific T cell repertoire, we analysed clones elicited by the lysine containing peptide $EYK(EYA)_3$. Since Type A clones are absolutely dependent on peptides containing lysine residues, and the $EYK(EYA)_3$ peptide contains a readily accessible lysine residue in the appropriate position, it seemed ideal for the elicitation of the Type A clones. We performed an antigen specificity analysis of EYK(EYA)$_3$ specific T cell hybridomas using the lysine free peptide (EYA)$_5$ and the lysine containing peptide K3K in the same manner as we analysed the Poly-18 specific T cell hybridomas. The results in Fig. 2.2b indicate that the ratio of (EYA)$_5$ specific to K3K specific T cell clones in this population is also approximatelly 2:1. Therefore, both Poly-18 and the lysine containing peptide EYK(EYA)$_3$ elicit Type B clones more effectively than they elicit Type A clones.

We also analysed whether the presence of lysine is actually necessary for the elicitation of strongly K3K reactive T cell clones since only the subpopulation represented by Type A clones is actually lysine dependent. We used the lysine free peptide (EYA)$_5$ to generate (EYA)$_5$ specific T cells and subsequently T cell hybridomas. We performed an antigen specificity analysis of these (EYA)$_5$ specific T cell hybridomas using the peptides (EYA)$_5$ and K3K as above. The results in Fig. 2.2c indicate that the lysine free peptide (EYA)$_5$ can also elicit strongly K3K reactive T cell clones, although only half as efficiently as the lysine containing peptide EYK(EYA)$_3$.

The Poly-18 specific T cell repertoire is larger than is detected by immunization with the whole Poly-18 molecule.

We used a series of alanine substituted (EYA)$_5$ peptides to further analyse the antigen specificity of T cells elicited by Poly-18. We find that Poly-18 specific T cells do not respond to (EYA)$_5$ peptides substituted with alanine at positions 8 [(EYA)$_5$sub8] or 11 [(EYA)$_5$sub11] while they do respond to (EYA)$_5$ peptides substituted with alanine at positions 4, 5, 7, and 10. (Fig. 2.3) We analysed the immunogenic and antigenic potential of the two peptides (EYA)$_5$sub8 and

$(EYA)_5$sub11 by assessing their ability to elicit Poly-18 and/or peptide specific T cells in vivo and to stimulate the elicited T cells to proliferate in vitro. (Table 2.5) The peptide $(EYA)_5$sub11 is only weakly immunogenic and elicits only a small number of $(EYA)_5$sub11 specific T cells and no Poly-18 specific T cells. Consequently peptide $(EYA)_5$sub11 lacks representation in the Poly-18 specific T cell repertoire. However, the peptide $(EYA)_5$sub8 is strongly immunogenic as well as strongly antigenic and elicits T cells specific for $(EYA)_5$sub8 as well as Poly-18 and the two peptides $(EYA)_5$ and $EYK(EYA)_4$. Furtheremore the peptides $(EYA)_5$ and $EYK(EYA)_4$, representing Poly-18 immunodominant epitopes, both elicit $(EYA)_5$sub8 specific T cell clones. (Table 2.5) The T cell clones specific for the peptide $(EYA)_5$sub8 thus appear to be elicited only when the Poly-18 immunodominant epitopes are used as immunogens in an isolated peptide form and not when they are present in the molecular context of the Poly-18 alpha helix.

We also used a series of alanine substituted K3K peptides to analyse the antigen specificity of the T cell clones elicited by Poly-18. We find that Poly-18 specific T cells do not respond to K3K peptides substituted with alanine at positions 4 and 5 [K3Ksub4-5], 7 and 8 [K3Ksub7-8] or 10 and 11 [K3Ksub10-11]. (Fig. 2.4) Analysis of the immunogenic and antigenic potential of these K3K substituted peptides indicates that they are all strongly immunogenic as well as antigenic and all elicit peptide specific T cells. However, only the peptide K3K and K3Ksub7-8 also elicit Poly-18 specific T cells. (Table 2.6) Thus, the clones specific for the peptide K3Ksub7-8 are also part of the Poly-18 repertoire but are greatly underrepresented when Poly-18 is used as the immunogen.

Discussion

All the potential sequential epitopes of the Poly-18 antigen have extensive amino acid sequence overlap as shown in Table 2.1. In spite of the apparent similarity between the various Poly-18 epitopes, BALB/c (H-2^d) derived Poly-18 specific T cells do not respond to all of them. The Poly-18 specific T cell response in BALB/c(H-2^d) mice is focused onto the lysine free epitope represented by $(EYA)_4$ or $(EYA)_5$ and the lysine containing epitope represented by $EYK(EYA)_3$ or $EYK(EYA)_4$. Peptides representing these two Poly-18 immunodominant epitopes are strongly immunogenic as well as strongly antigenic.

Poly-18 also contains a subdominant epitope represented by the C-terminal lysine containing peptides $(EYA)_3EYK$ or $(EYA)_3EYKEYA$. Subdominant epitopes have been described in the HEL system as epitopes to which no detectable response is generated when the whole HEL molecule is used as an immunogen. However, when the subdominant epitope is used as an immunogen it elicits T cell responses specific for itself as well as the whole HEL molecule [26,34]. The peptides representing the Poly-18 subdominant epitope are similarly able to elicit Poly-18 specific T cell responses but peptide specific T cell responses are difficult to detect since these peptides appear to be very poorly antigenic in vitro.

Poly-18 epitopes which contain lysine residues in a more central location within the epitope such as $EYAEYK(EYA)_2$, $(EYA)_2EYKEYA$, or $(EYA)_2EYK(EYA)_2$ do not appear to be immunogenic or antigenic and Poly-18 specific T cells do not respond to them. Thus there is a clear dominance relationship between the various Poly-18 epitopes depending on the location of the lysine residue within the epitopes.

We analysed the antigen specificity of the Poly-18 specific T cells which are directed at the two Poly-18 immunodominant epitopes. Poly-18 elicits two T cell clonotypes, Type A and Type B, in BALB/c(H-2^d) mice [31]. The specificity of Type A clones is very restricted while the specificity of Type B clones is broader. T cell hybridomas representing Type A clones are absolutely dependent on the presence of lysine in position 3 and respond to the Poly-18 derived peptide EYK$(EYA)_4$. On the other hand, T cell hybridomas representing Type B clones are lysine independent and respond to the Poly-18 derived peptide $(EYA)_5$. However, Type B T cell hybridomas also respond to the peptide EYK$(EYA)_4$, containing a lysine residue in position 3, and to a lesser extent also to the peptide $(EYA)_4$EYK, containing a lysine residue at the C-terminus in position 15 and representing the subdominant epitope of Poly-18. Although all Type B T cell hybridomas respond to peptides containing lysine residues at either position 3 or 15 they vary in their ability to respond to the peptide EYK$(EYA)_3$EYK [Abbr. K3K] which contains lysine at both positions 3 and 15 while all lysine dependent Type A clones respond to this peptide.

We used the K3K peptide and the $(EYA)_5$ peptide to show that the Poly-18 specific T cell clones actually represent a spectrum of clones with more or less ability to respond to these two peptides. Clones responding only to $(EYA)_5$ or $(EYA)_5$ and poorly to K3K are considered to have a lot of Type B character while clones responding only to K3K or K3K and poorly to $(EYA)_5$ are considered to have a lot of Type A character. By choosing appropriately low concentrations (10 μM) of the $(EYA)_5$ and K3K peptides we assessed the relative frequency of strongly reactive $(EYA)_5$ specific T cell clones, to strongly reactive K3K specific T cell clones in the Poly-18 specific T cell repertoire.

Strongly reactive $(EYA)_5$ specific T cell clones represent almost all Type B clones while strongly reactive K3K specific T cell clones represent all Type A clones as well as a subpopulation of Type B clones (Refer to Table 2.4). Our analysis indicates that the Poly-18 specific T cell repertoire contains only half as many K3K specific T cell clones relative to $(EYA)_5$ specific T cell clones. Since the K3K specific T cell clones do not represent only Type A clones but also the strongly K3K reactive subpopulation of Type B clones, this analysis clearly indicates that the Type B clones are much more frequent than the Type A clones in the Poly-18 specific T cell repertoire.

The dominance of Type B clones could be related to the degeneracy of their antigen specificity. Type B clones could potentially be elicited by at least 3 Poly-18 epitopes ie. $(EYA)_4$, $EYK(EYA)_3$ and $(EYA)_3EYK$ while Type A clones can be elicited only by the Poly-18 epitope $EYK(EYA)_3$. The dominance of Type B clones could therefore be related purely to the availability of appropriate epitopes. However, we observe the dominance of Type B clones even when the lysine containing peptide $EYK(EYA)_3$, representing only the lysine containing Poly-18 immunodominant epitope, is used as the immunogen. This suggests that the Type B clones actually represent a more numerous clonotype than the Type A clones in the unimmunized peripheral T cell repertoire of BALB/c mice.

Since the Type B clonotype can be separated into strongly K3K reactive and weakly K3K reactive subpopulations we asked whether the lysine free peptide $(EYA)_5$ can elicit the strongly K3K reactive Type B subpopulation. Our analysis indicates that the $(EYA)_5$ peptide elicits approximatelly half as many K3K specific T cell clones as the lysine containing antigens $EYK(EYA)_3$ or Poly-18. This result clearly demonstrates that the presence of lysine is not necessary for the elicitation of the strongly K3K reactive Type B clones. The $(EYA)_5$ peptide therefore elicits a spectrum of $(EYA)_5$ specific T cells with variable abilities to crossreact with the lysine containing peptide K3K. Our extensive antigen specificity analysis of one Type B T cell hybridoma B.1.1 (B15) indicates that the critical amino acid residues necessary for the activation of this T cell hybridoma reside near the centre of the $(EYA)_5$ epitope at positions 6,7,8,9,11 and 12 (M.Boyer et al. manuscript in preparation). Since almost all Type B T cell hybridomas respond to the K3K peptide, although some only at very high antigen concentrations (Table 2.4), it suggests that the critical amino acid residues necessary for the activation of all these $(EYA)_5$ specific T cell clones are also located near the centre of the $(EYA)_5$ epitope. The amino acid residues near the N- and C-terminus of the $(EYA)_5$ epitope can therefore be substituted with residues such as lysine without affecting the ability of these T cell hybridomas to respond. This is most likely the basis for the apparently degenerate recognition of lysine containing peptides such as $EYK(EYA)_4$, $(EYA)_4EYK$ and K3K by $(EYA)_5$ specific Type B T cell clones.

Another level of epitope dominance concerns epitopes which are related to the Poly-18 immunodominant epitopes ie. alanine substituted peptides of $(EYA)_5$ or K3K. Some of these epitopes have a clear potential to be part of the Poly-18 specific T cell repertoire in BALB/c mice and yet are greatly under represented. The $(EYA)_5$ epitope substituted with alanine at position 8 [$(EYA)_5$sub8] is strongly immunongenic as well as antigenic and elicits T cell responses specific not only for itself but also the two immunodominant peptides $(EYA)_5$ and $EYK(EYA)_4$ as well as the Poly-18 antigen. In addition, the two immunodominant peptides $(EYA)_5$ and $EYK(EYA)_4$ both elicit T cell responses specific for the $(EYA)_5$sub8 peptide. However, the Poly-18 antigen does not elicit detectable T cells capable of responding to this peptide. This ability of peptides to elicit a broader range of T cell specificities compared to that elicited by whole molecules has also been observed in the myoglobin [35] and HEL [26,34] antigen systems. In addition, the choice between two distinct T cell determinants within the 23 amino acid region 74-96 of HEL has been shown to depend on their structural context ie. native or denatured [36]. It therefore appears that different epitopes are the major focus of the immune response depending on the molecular context within which those epitopes are presented. This is a clear indication that antigen processing plays a critical role in epitope selection. Undoubtedly the size of the epitopes, their amino acid boundaries and potential structural features are different when an antigen is processed or fragmented in vivo compared to the uniform synthetic fragments that we generate in vitro. By immunizing with well defined synthetic peptides we are introducing a high concentration of particular epitopes which may be generated relatively infrequently or not at all during in vivo antigen processing. For instance, in vivo differential antigen processing of species variant native lysozyme molecules but not lysozyme fragments appears to result in the heteroclicity of B6 clones for ring necked pheasant lysozyme(REL) in preference to HEL [37].

Although we have shown that the minimum peptides of Poly-18 do not require to be processed prior to presentation to T cell hybridomas [30], it is still possible that the T cell clones specific for highly immunogenic epitopes, such as $(EYA)_5$sub8 or K3Ksub7-8 which elicit peptide specific as well as Poly-18 specific T cells, may not be represented in the Poly-18 specific T cell repertoire of BALB/c mice due to biases in antigen processing. Fox et al. [38] have shown that the further processing of a minimal cytochrome c peptide which did not require to be processed for recognition by cytochrome c specific T cells alters its interaction with MHC molecules. However, antigen processing biases are not likely to be the reason why epitopes such as $EYAEYK(EYA)_2$, $(EYA)_2EYKEYA$ or $(EYA)_2EYK(EYA)_2$ are not represented in the Poly-18 specific T cell repertoire. These epitopes appear to be nonimmunogenic even when high concentrations of the $EYAEYK(EYA)_2$, $(EYA)_2EYKEYA$ or $(EYA)_2EYK(EYA)_2$ peptides are used for immunization. Under these circumstances, epitope specific nonresponsiveness can occur for several reasons.

Firstly, the failure to positively select for appropriate antigen specific self MHC restricted T cells in the thymus or the intrathymic deletion of maturing T cell clones that are reactive to self determinants, which is necessary for the maintenance of self tolerance, may lead to functional holes in the expressed T cell repertoire [14-17]. The $(EYA)_2EYK(EYA)_2$ epitope, which encompasses the epitopes $EYAEYK(EYA)_2$ and $(EYA)_2EYKEYA$, may thus fortuitously resemble some self antigen of BALB/c mice resulting in the functional elimination of $(EYA)_2EYK(EYA)_2$ specific T cell clones from their expressed T cell repertoire. Although all the Poly-18 epitopes have extensive amino acid sequence homology, tolerance induction has been shown to be highly epitope specific. Neonatal tolerance studies using three cytochrome c derived peptides which differ by only one amino acid each have shown that tolerance can be induced to each individual peptide without severely affecting responses to the other two peptides [39]. Tolerance induction could therefore be specifically targeted at the $(EYA)_2EYK(EYA)_2$ epitope without affecting the T cell responses directed at the remaining Poly-18 epitopes.

The basis for such exquisite epitope specific tolerance induction is most likely related to the presence of epitope specific clonal hierarchies. Even very closely related peptides such as the aforementioned cytochrome c derived peptides [39] or our own alanine substituted K3K peptides K3Ksub4-5, K3Ksub7-8 and K3Ksub10-11 are the focus of very different T cell specificities. Although all three of our alanine substituted peptides are highly immunogenic and elicit peptide specific T cells, only the peptide K3K and K3Ksub7-8 select for T cells which can also respond to the Poly-18 antigen. Therefore, only epitopes which are identical to self epitopes and share identical clonal hierarchies would be rendered nonimmunogenic by some mechanism of self tolerance while even very closely related epitopes would likely remain unaffected or be only minimally affected.

Secondly, since T cells do not respond to soluble antigen but rather recognize antigen only in association with self MHC molecules on the surface of antigen presenting cells (APC), some epitopes may not have the ability to bind to self Ia molecules and will thus not be recognized by available T cells. One line of evidence which suggests that the $(EYA)_2EYK(EYA)_2$ peptide may have problems associating with H-2^d Ia molecules involves our observation that this peptide does not competitively inhibit the activation of the Type B T cell hybridoma B.1.1(B15) by peptides such as $(EYA)_5$ or $EYK(EYA)_4$. However, only direct binding studies between the $(EYA)_2EYK(EYA)_2$ peptide and the H-2^d Ia molecules can answer this question definitively. Direct binding studies between Ia molecules of various mouse strains and ovalbumin [40] or HEL [41] derived peptides demonstrated that the peptides bound efficiently only to their respective restricting Ia molecules and not to Ia molecules which do not present the peptides.

Finally, regulatory T suppressor cells may influence epitope specific non responsiveness. Epitopes of several antigens including lysozyme [42,43], beta-galactosidase [43,44], poly-(Glu,Ala,Tyr) [45] and insulin [46] have been shown to induce suppressor T cells in selected strains of mice. Although some of the elicited T suppressor cells are broadly suppressive others appear to have a more epitope specific suppressive activity. We did not address the question whether epitope specific suppression is responsible for the apparent nonimmunogenicity of the $(EYA)_2EYK(EYA)_2$ Poly-18 epitope. However, our analysis of Poly-18 nonresponder mouse strains failed to reveal any evidence of T cell mediated suppression [28].

Table 2.1. Sequential overlapping 12 and 15 amino acid long epitopes of Poly-18.

Peptide	Abbreviation	Amino Acid Sequence											
Poly-EYK(EYA)$_5$	Poly-18	EYK	EYA	EYA	EYA	EYA	EYA	EYK	EYA	EYA	EYA	EYA	EYA..
EYK(EYA)$_3$	K3	EYK	EYA	EYA	EYA								
(EYA)$_4$	E4		EYA	EYA	EYA	EYA							
				EYA	EYA	EYA	EYA						
(EYA)$_3$EYK	3K				EYA	EYA	EYA	EYK					
(EYA)$_2$EYK EYA	2K1					EYA	EYA	EYK	EYA				
EYA EYK(EYA)$_2$	1K2						EYA	EYK	EYA	EYA			
EYK(EYA)$_4$	K4	EYK	EYA	EYA	EYA	EYA							
(EYA)$_5$	E5		EYA	EYA	EYA	EYA	EYA						
(EYA)$_4$EYK	4K			EYA	EYA	EYA	EYA	EYK					
(EYA)$_3$EYK EYA	3K1				EYA	EYA	EYA	EYK	EYA				
(EYA)$_2$EYK(EYA)$_2$	2K2					EYA	EYA	EYK	EYA	EYA			
EYA EYK(EYA)$_3$	1K3						EYA	EYK	EYA	EYA	EYA		

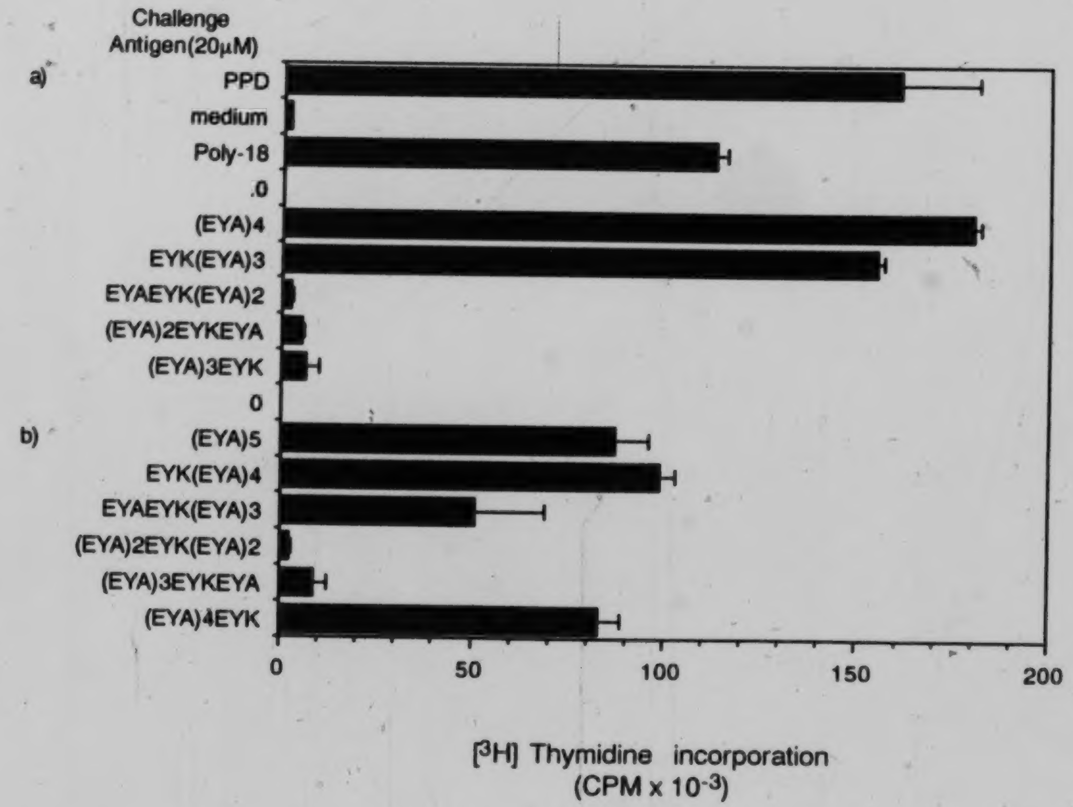

Figure 2.1. In vitro proliferative responses of Poly - 18 primed BALB/c lymph node T cells to 12 and 15 amino acid long Poly - 18 derived peptides. For these assays 6.5 x 10^5 Poly - 18 primed viable lymph node cells were cultured in the presence of 20μM antigen. See Materials and Methods for details. Results represent the mean of duplicate cultures +/- S.D.

Table 2.2. In vitro proliferative response of peptide primed BALB/c lymph node T cells to Poly - 18 and the priming peptides.

Priming Antigen[a]	[^{3}H] Thymidine incorporation[b] (CPM x 10^{-3}) Challenge antigen (20µM)			
	PPD	Medium	Poly - 18	Priming Antigen
$(EYA)_4$	130 +/- 5	7.1 +/- 0.5	88 +/- 12	69 +/- 6
$EYK(EYA)_3$	186 +/- 32	6.7 +/- 2.1	144 +/- 35	64 +/- 16
$EYA\ EYK(EYA)_2$	184 +/- 5	1.1 +/- 0.5	1 +/- 0.1	1 +/- 0.1
$(EYA)_2EYK\ EYA$	147 +/- 10	1.2 +/- 0.1	8 +/- 0.2	2 +/- 0.5
$(EYA)_3EYK$	162 +/- 6	3.5 +/- 0.2	48 +/- 8	9 +/- 3
$(EYA)_5$	153 +/- 6	2.0 +/- 0.5	132 +/- 9	139 +/- 12
$EYK(EYA)_4$	231 +/- 32	1.1 +/- 0.3	95 +/- 31	119 +/- 31
$(EYA)_2EYK(EYA)_2$	110 +/- 12	1.5 +/- 0.2	1 +/- 1.4	1 +/- 0.2
$(RYA)_3EYK\ EYA$	84 +/- 9	1.9 +/- 1.2	11 +/- 3	2 +/- 0.3
$(EYA)_4EYK$	146 +/- 7	7.5 +/- 3.2	105 +/- 17	68 +/- 21

a. Peptides from Table 2.1.

b. 6.5 x 10^5 peptide primed viable lymph node T cells were cultured in the presence of 20 µM antigen.. See Materials and Methods for details. Results represent the mean of triplicate cultures +/-S.D.

Table 2.3. Antigen reactivity patterns of Poly - 18 specific Type A and Type B T cell hybridomas

Antigen	Abbreviation	Ag Dose µM	TYPE A[a] (CPM x 10^{-3})[c]	TYPE B[b]
EYA EYA EYA EYA EYA	E5	10	1-3	>50
EYK EYA EYA EYA EYA	K4	10	>50	>50
EYA EYA EYK EYA EYA	2K2	10	1-3	1-3
EYA EYA EYA EYA EYK	4K	10	1-3	10-20
EYK EYA EYA EYA EYK	K3K	10	>50	*
		100	>50	*

a. Responses of a representative Type A hybridoma A.1.1(31).
b. Responses of representative Type B hybridomas B15 and B16 (Chapter 4).
c. 10^5 T cell hybridomas were cultured with antigen and 10^6 irradiated (4000R) BALB/c spleen cells as APC. Supernatants from 24 hr cultures were tested for their ability to stimulate [^{3}H] - thymidine uptake by 10^4 CTLL cells. (See Materials and Methods for details)
* Response varies depending on the Type B hybridoma used. See Table 2.4.

Table 2.4. Responses of Poly - 18 specific T cell hybridomas to $(EYA)_5$ and K3K peptides.

Poly - 18 specific T cell hybridomas		Antigen specific responses [a] (CPM x 10^{-3})				SUMMARY of antigen specific responses.		
		$(EYA)_5$		K3K				
		10μM	100μM	10μM	100μM	$(EYA)_5$ reactivity	K3K reactivity	
TYPE B [b]	(i)	1-3	>50	1-3	1-3	Low	none	a
	(ii)	>50	>50	1-3	>50	High	Low	
	(iii)	>50	>50	>50	>50	High	High	b
	(iv)	1-3	>50	>50	>50	Low	High	
TYPE A [c]		1-3	1-3	>50	>50	none	High	

a - Weakly K3K reactive
b - Strongly K3K reactive

a. Responses were measured as in Table 2.3.
b. Responses of representative Type B hybridomas (i) B4, (ii) B15, (iii) B16, (iv) B17 (Chapter 4).
c. Responses of a representative Type A hybridoma A.1.1(31).

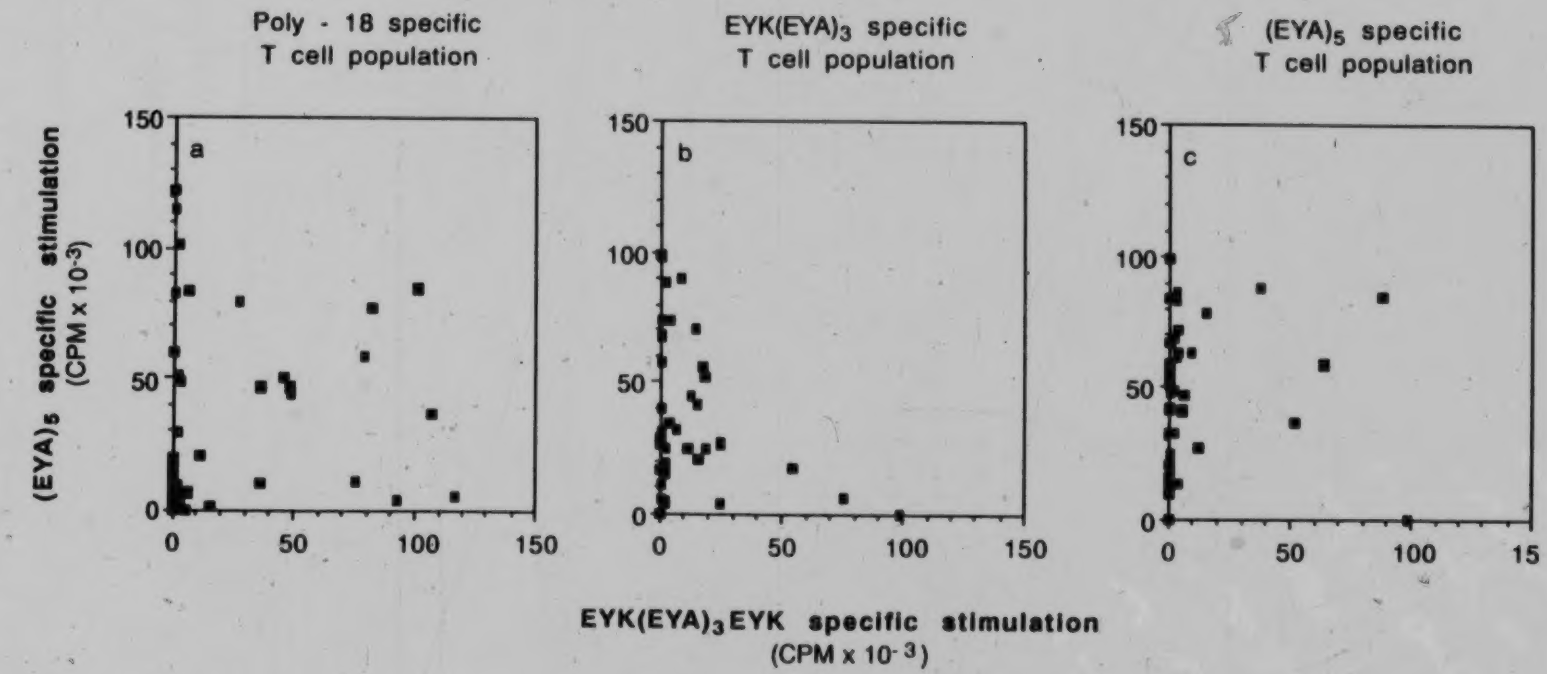

Figure 2.2. Relative frequency of (EYA)$_5$ specific and K3K specific T cell clones in various T cell populations. The antigen specificity of T cell hybridomas using the antigens EYK(EYA)$_3$EYK and (EYA)$_5$ was tested as described in Materials and Methods. CTLL medium control backgrouds were identical to hybridoma plus APC medium control backgrounds, ranging from 1000-3000 CPM. Medium control backgrounds were subtracted from antigen specific stimulations which were then plotted in an (EYA)$_5$ specific stimulation (ordinate) versus EYK(EYA)$_3$EYK specific stimulation (abscisa) matrix. The number of linbro wells tested for each T cell line was (a) 48 in the Poly-18 specific T cell line, (b) 33 in the EYK(EYA)$_3$ specific T cell line and (c) 39 in the (EYA)$_5$ specific T cell line.

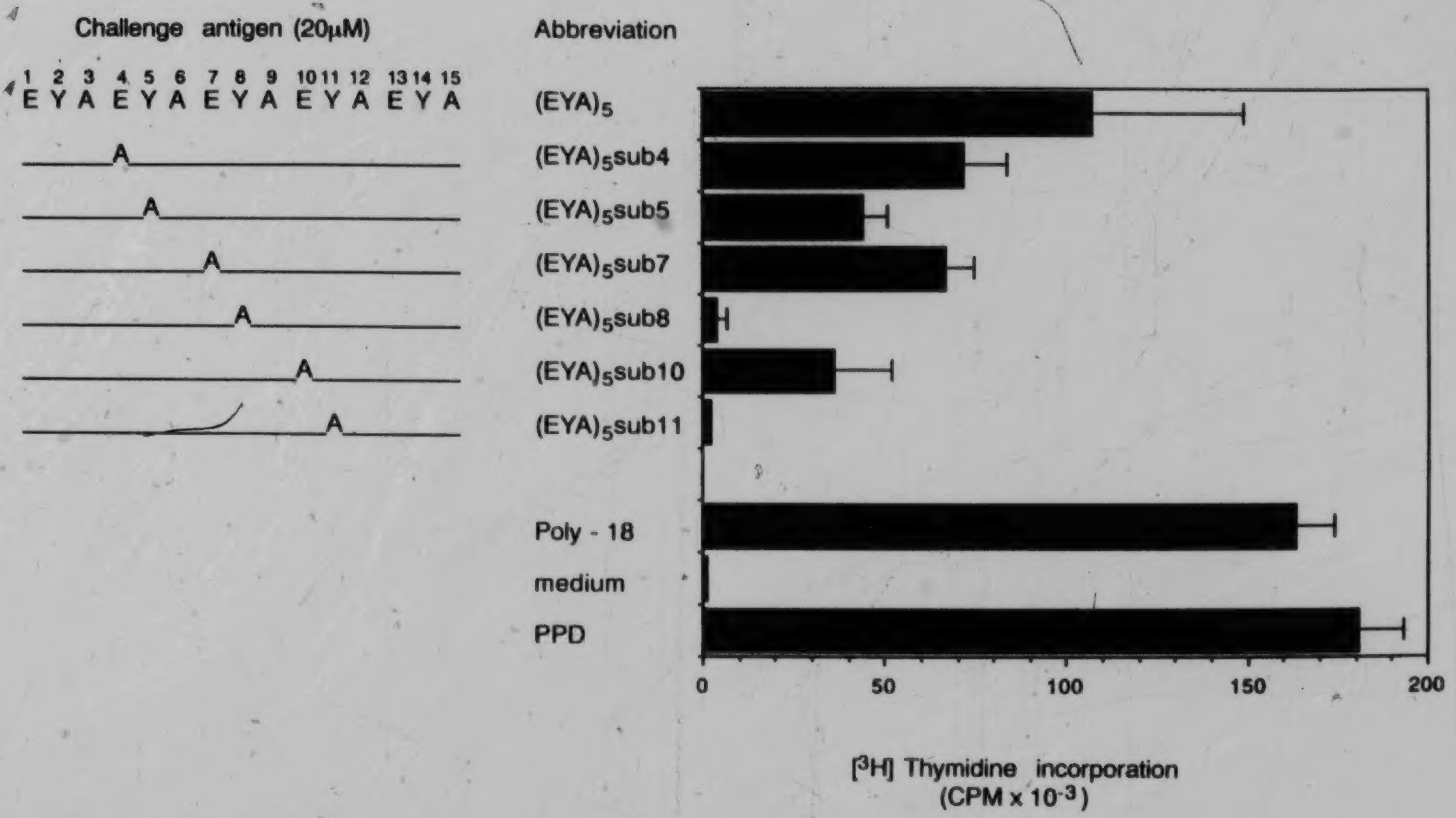

Figure 2. 3. In vitro Proliferative responses of Poly - 18 primed BALB/c lymph node T cells to alanine substituted $(EYA)_5$ peptides. Responses were measure as in Fig 2.1. Results represent the mean of triplicate cultures +/- S.D.

Table 2.5. In vitro proliferative responses of (alanine substituted $(EYA)_5$ peptides). EYA_5 and $EYK(EYA)_4$ primed BALB/c lymph node T cells to Poly - 18 and the priming peptides.

Priming [a] Antigen	[^{3}H] Thymidine incorporation [b] (CPM x 10^{-3}) Challenge antigen (20 μM.)						
	$(EYA)_5$sub8	$(EYA)_5$sub11	$(EYA)_5$	$EYK(EYA)_4$	Poly-18	medium	PPD
$(EYA)_5$sub8	142 +/- 12	40 +/- 10	144 +/- 5	85 +/- 7	62 +/- 20	1.9 +/- 0.2	200 +/- 6
$(EYA)_5$sub11	25 +/- 6	33 +/- 7	17 +/- 2	27 +/- 14	7 +/- 2	0.9 +/- 0.2	158 +/- 7
$(EYA)_5$	57 +/- 11	31 +/- 12	139 +/- 12	119 +/- 8	132 +/- 9	2.0 +/- 0.5	153 +/- 6
$EYK(EYA)_4$	87 +/- 17	57 +/- 25	138 +/- 15	119 +/- 31	95 +/- 31	1.1 +/- 0.3	231 +/- 33

a. Peptides from fig 2.3.
b. Responses were measured as in Table 2.2. Results represent the mean of triplicate cultures +/-S.D.

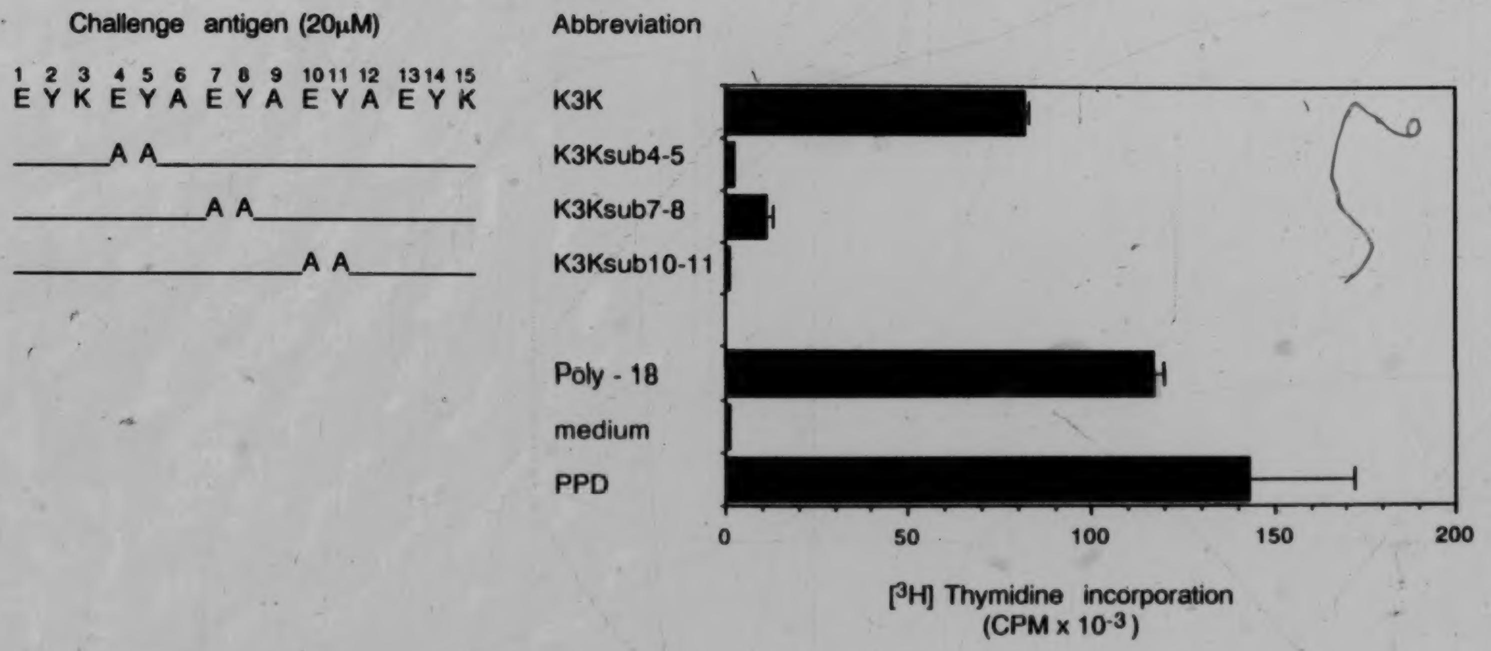

Figure 2. 4. In vitro Proliferative responses of Poly - 18 primed BALB/c lymph node T cells to alanine substituted K3K peptides. Responses were measure as in Fig 2.1. Results represent the mean of duplicate cultures +/- S.D.

Table 2.6. In vitro proliferative responses of peptide (K3K and alanine substituted K3K) primed BALB/c lymph node T cells to Poly - 18 and the priming peptides.

Priming [a] Antigen	$[^3H]$ Thymidine incorporation [b] (CPM x 10^{-3}) Challenge antigen (20μM)			
	Priming antigen	Poly - 18	medium	PPD
K3K	79 +/- 12	83 +/- 7	3.0 +/-	0.9195 +/- 11
K3Ksub4-5	52 +/- 2	3 +/- 2	1.1 +/-	0.3162 +/- 4
K3Ksub7-8	96 +/- 2	46 +/- 8	3.8 +/-	0.4218 +/- 32
K3Ksub10-11	59 +/- 6	5 +/- 1	2.0 +/-	0.1187 +/- 3

a. Peptides from fig 2.4.

b. Responses were measured as in Table 2.2. Results represent the mean of duplicate cultures +/- S.D.

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CHAPTER 3

Analysis of Poly-18 specific T cell clonotypes in normal Responder and Nonresponder->(Responder x Nonresponder)F1 bone marrow radiation chimeric mice

(Paper II)

Introduction

The immune system has the capacity to generate a diverse T cell repertoire capable of recognizing almost any foreign antigen in the context of self major histocompatibility complex (MHC) gene products [1,2]. Unresponsiveness to a particular antigen or Ir gene defect [3,4] maps to the I-region of the mouse MHC [5,6] and may be due to deletion of the T cell clonotypes because of cross-reactivity with self antigens [7,8] or the failure of nonresponder Ia gene products to present antigen to available T cells [9-11]. T cell precursors from nonresponder mice can be educated in a reponder environment to recognize a particular antigen in the context of the responder I-region gene products [12-14]. This suggests that there are no limitations in the ability of nonresponder T cells to generate a full range of T cell specificities.

We educated T cell precursors from Poly-18, Poly EYK(EYA)$_5$, nonresponder H-2^b mice [15] in a responder H-2^{bxd} environment to recognize Poly-18, a synthetic alpha-helical polypeptide antigen under Ir-gene control, in the context of the responder H-2^d I-region gene products. The use of sythetic fragments representing the dominant epitopes of Poly-18 enabled us to analyse the full range of H-2^d restricted T cell specificities generated by nonresponder T cells at the clonal level. Two major categories of I-A^d restricted T cell clones, EYK(EYA)$_3$EYK specific and (EYA)$_5$ specific, are induced by Poly-18 in H-2^d responder animals [16]. Similarly, both EYK(EYA)$_3$EYK specific and (EYA)$_5$ specific T cell clones are induced by Poly-18 in H-2^{bxd} and H-2^{bxd}->H-2^{bxd} responder animals. H-2^b T cells from H-2^b->H-2^{bxd} responder animals fail to generate the EYK(EYA)$_3$EYK specific T cell clones even though the H-2^{bxd} environment is permissive for both EYK(EYA)$_3$EYK specific and (EYA)$_5$ specific T cell clones and also provides the responder Class II MHC determinants for their selection.

Materials and Methods

Mice- C57BL/6 (B6, H-2^b), BALB/c(H-2^d) and (BALB/c x C57Bl/6)F1 (H-2^{bxd}) mice were obtained from The Jackson Laboratory, Bar Harbor, ME.

Peptides- Poly-18 and the peptides $(EYA)_5$ and $EYK(EYA)_3EYK$ were synthesized by the Merrifield solid phase peptide synthesis technique as described previously [17-19]. K, lysine; E, glutamic acid; Y, tyrosine; A, alanine.

Bone marrow radiation chimeras- (BALB/c x C57BL/6)F1 mice were irradiated with 1000 R and reconstituted 24 hrs later with an intravenous injection of anti-Thy 1.2 plus complement treated 10^7 (BALB/c x C57BL/6)F1 or C57BL/6 bone marrow(BM) cells. 3-6 months later the chimerism was analysed using a Glucose phosphate isomerase (GPI) assay [20] which measures the presence of strain specific isozymes. The sensitivity of the GPI assay is 2%. All chimeric animals survived and had no signs of graft versus host disease (GVHD). All were 100% donor in their lymph nodes and peripheral red blood cells 3 months after bone marrow transplantation and were used for antigen priming 4-6 months post transplantation.

T cell lines- Five normal BALB/c, 5 normal (BALB/c x C57BL/6)F1, 6 F1->F1 chimeras, 10 C57BL/6->F1 chimeras (Line I), 7 C57BL/6->F1 chimeras (Line II) and 6 C57BL/6->F1 chimeras (Line III) were primed in both hind footpads with 30μg of Poly-18 in complete freunds adjuvant(CFA). 7-9 days later lymph node T cells were nylon wool separated and $2\text{-}4\times10^5$ T cells/ml were cultured with 3×10^6 irradiated(4000 R) spleen cells/ml and 10μg Poly-18/ml in RPMI 1640 (Gibco) containing 10% fetal bovine serum(FCS), 5×10^{-5} M 2-mercaptoethanol, 10 mM Hepes, 2 mM glutamine and penicillin-streptomycin. T cell lines from normal mice were carried in the presence Poly-18 and syngeneic irradiated spleen cells while T cell lines from chimeric mice were carried in the presence of Poly-18 and

(BALB/c x C57BL/6)F1 irradiated spleen cells for 1-4 months prior to fusing with BW5147 thymoma cells. T cells from chimeric mice were analysed using the GPI assay and phenotyped as 100% donor when isolated freshly from Poly-18 primed lymph nodes as well as after expansion in long term Poly-18 specific T cell lines.

T cell proliferation- T cells from Poly-18 specific T cell lines were cocultured with 4×10^5 BALB/c (H-2^d) irradiated (4000 R) spleen cells as APC and 10μM antigen in 300μl of T cell line medium. Cultures were pulsed with [^{3}H]-thymidine 3.5 days later for 4-6 hours prior to harvesting.

T cell fusions- T cell fusions were performed as previously described using a standard PEG fusion protocol [16] and the standard AKR thymoma line BW5147 or BW5147α−β-, a derivative of BW5147 which does not express the alpha and beta chains of the T cell receptor, which was kindly made available to us by Drs. P. Marrack & J. Kappler, Denver. After fusion, cells were plated in 24 well linbro plates at 2×10^5 BW5147 cells/well. T cell hybridomas were initially grown in Hypoxanthine-Thymidine medium for two weeks and eventually adapted to drug free medium containing only RPMI 1640 and 10% FCS. Hybridomas were then tested for IL-2 release in the presence of various antigens and irradiated (4000 R) spleen cells as APC or subcloned by limiting dilution prior to testing.

T cell hybridoma antigen specificity analysis- T cell hybridomas from individual linbro wells (Fig 3.1) or recloned T cell hybridomas (Table 3.1 and 3.4) were incubated at 10^5 cells/well with (10^6) BALB/c (H-2^d) irradiated (4000 R) spleen cells and 10μM antigen in 300μl RPMI 1640 and 10% FCS for 24 hours. The supernatants were diluted at 1:2 in 200μl and tested for the presence of IL-2 by their ability to support the growth of 10^4 IL-2-dependent CTLL cells, as measured by ^{3}H-thymidine incorporation.

Results

Poly-18 specific T cell clonotypes in BALB/c mice.

The antigen specificity of Poly-18 specific, BALB/c (H-2^d) derived, T cell hybridomas indicates that EYK(EYA)$_3$EYK [Abbr. K3K] specific clones recognize lysine containing fragments of Poly-18 ie. EYK(EYA)$_4$ while (EYA)$_5$ specific clones recognize the lysine-free part of the Poly-18 molecule. The K3K specific and (EYA)$_5$ specific clones are represented by three distinct T cell clonotypes (Chapter 2) [16]. (Table 3.1) One clonotype responds only to peptides containing lysine residues ie. EYK(EYA)$_4$ and EYK(EYA)$_3$EYK (Type A- K3K specific). The second clonotype responds to the lysine free peptide (EYA)$_5$ and crossreacts on EYK(EYA)$_4$ and (EYA)$_4$EYK but not on EYK(EYA)$_3$EYK (Type B-(EYA)$_5$ specific). The third clonotype responds to the lysine free peptide (EYA)$_5$ and crossreacts on EYK(EYA)$_4$ and (EYA)$_4$EYK as well as EYK(EYA)$_3$EYK (Type B- K3K and (EYA)$_5$ specific). This third clonotype therefore contributes equally to the enumeration of K3K specific as well as (EYA)$_5$ specific clones when EYK(EYA)$_3$EYK and (EYA)$_5$ are used as the diagnostic antigens for the two categories of clones, respectively.

Relative frequency of (EYA)$_5$ and K3K specific T cell clones in the Poly-18 specific T cell repertoire of normal BALB/c and (BALB/c x C57BL/6)F1 mice.

We generated Poly-18 specific T cell lines from normal BALB/c and (BALB/c x B6)F1 responder animals (Table 3.2) and the ability of these lines to respond to Poly-18 in the context of H-2^d MHC gene products is demonstrated in Table 3.3. These Poly-18 specific H-2^d restricted T cell lines were used to generate T cell hybridomas. The results in Figure 3.1a indicate that the ratio of $(EYA)_5$ specific to K3K specific clones in normal BALB/c mice is approximately 2:1 in the T cell hybridoma population prior to subcloning. In normal (BALB/c x B6)F1 ($H\text{-}2^{bxd}$) mice, the ratio of $H\text{-}2^d$ restricted $(EYA)_5$ specific to K3K specific clones is also approximately 2:1 as shown in Figure 3.1b. Thus, the presence of the nonresponder $H\text{-}2^b$ gene products in the $H\text{-}2^{bxd}$ environment does not appear to exert any influence on the generation of appropriate Poly-18 specific clonotypes.

Relative frequency of $(EYA)_5$ and K3K specific T cell clones in the Poly-18 specific T cell repertoire of C57BL/6->(BALB/c x C57BL/6)F1 and (BALB/c x C57BL/6)F1->(BALB/c x C57BL/6)F1 chimeric mice.

Poly-18 specific T cell clonotypes in B6->(BALB/c x B6)F1 ($H\text{-}2^b$->$H\text{-}2^{bxd}$) bone marrow radiation chimeras were next investigated. Three independent Poly-18 specific b->F1 T cell lines (Line I, II and III) were established and fused separately to generate T cell hybridomas. (Table 3.2) Genotypic analysis of the T cells from Lines I, II and III indicated that they were all of $H\text{-}2^b$ origin. Their ability to respond to Poly-18 in the context of $H\text{-}2^d$ MHC gene products and lack of reactivity to $H\text{-}2^b$ APC is demonstrated in Table 3.3. Identical MHC restriction results were obtained with a panel of T cell hybridomas derived from the line b->F1 Line I. (Table 3.4) All of the hybridomas analysed are Poly-18 specific and $H\text{-}2^d$ restricted while none of them are $H\text{-}2^b$ restricted. When $H\text{-}2^b$ reactivity is observed it appears to be in the form of autoreactivity in that it is independent of the Poly-18 antigen. The contribution of the $H\text{-}2^b$ reactive T cell clones to the overall Poly-18 specific, $H\text{-}2^d$ restricted, response is not clear since the bulk T cell population from which these hybridomas were derived, b->F1

Line I, does not appear to have a general H-2^{b} reactivity. (Table 3.3) Frequency analysis of the (EYA)$_5$ specific and K3K specific clones in chimeric mice suggests an exclusive usage of (EYA)$_5$ specific clones and a complete absence of K3K specific clones. (Fig. 3.1c,d and e) To rule out the artifactual effects of irradiation on the selection of the clonotypes we generated (BALB/c x B6)F1->(BALB/c x B6)F1 (H-2^{bxd}->H-2^{bxd}) bone marrow radiation chimeras. (Table 3.2 and 3.3) Poly-18 specific T cell hybridomas generated from these chimeras demonstrate that the ratio of (EYA)$_5$ specific to K3K specific clones is approximately 2:1, shown in Figure 3.1f, and is identical to that found in unirradiated H-2^{bxd} animals.

Discussion

The inability to present antigen in the context of MHC gene products,being due to determinant selection, is excluded as a possible cause for the absence of K3K specific clones in H-2^b->F1 animals since Poly-18 specific responses are restricted to responder I-A^d. We did not find it necessary to infuse irradiated F1 H-2^{bxd} spleen cells, to function as antigen presenting cells, into our H-2^b->F1 chimeras in order to obtain antigen specific responses restricted to the responder H-2^d MHC gene products [21,22]. The expansion of H-2^d restricted, $(EYA)_5$ specific, clones in the H-2^b->F1 animals indicates that long lived F1 radio resistant Ia positive antigen presenting cells (APC), although present in low numbers relative to the reconstituting H-2^b bone marrow derived APC, are sufficient for in vivo priming against the Poly-18 antigen. Although we did find that a subpopulation of the H-2^d restricted, Poly-18 specific, T cell clones are autoreactive on H-2^b APC and could thus have been elicited fortuitously, most of the H-2^d restricted T cell clones do not appear to crossreact on H-2^b APC. In addition, we did not find any H-2^d restricted, Poly-18 specific clones in the H-2^b->F1 animals which were concomitantly H-2^b restricted and Poly-18 specific, confirming the importance of F1 H-2^{bxd} MHC gene products in the expansion of such clones.

The F1 radioresistant APC in the H-2^b->F1 animals should be equally capable of expanding both K3K specific and $(EYA)_5$ specific T cell clones. Antigen dose response analyses of our hybridomas indicate that there are no differences in the handling or presentation of lysine-free or lysine containing Poly-18 related peptides by normal or glutaraldehyde fixed I-A^d positive APC, suggesting that the two types of antigenic determinants do not have any distinguishing antigen processing requirements [23]. However, the two types of antigenic determinants may have differential antigen processing requirements in vivo and the limiting numbers of (H-2^{bxd})F1 APC in the H-2^b->F1 chimeras may ultimately be responsible for the phenotypic differences observed between the H-2^b->F1 and BALB/c, (C57BL/6 x BALB/c)F1 and (C57BL/6 x BALB/c)F1->(C57BL/6 x BALB/c)F1 Poly-18 specific T cell repertoires.

Excluding determinant selection, the epitope specific nonresponsiveness of H-2^b->F1 animals is most consistent with a hole in the T cell repertoire. Firstly, intrathymic deletion of autoreactive T cell clones is one way the immune system maintains a state of self tolerance [24-29]. The absence of K3K specific clones in the H-2^b->H-2^{bxd} F1 animals primed with Poly-18 is not likely to be due to such tolerance considerations since the normal and irradiated H-2^{bxd} F1 environment is permissive for both K3K specific and $(EYA)_5$ specific T cell clones. However, it is possible that the H-2^b donor cells are expressing a parent specific antigen which is not expressed by the heterozygous H-2^{bxd} F1 cells of the recipient and which crossreacts with the K3K determinant.

Secondly, since the H-2^b->F1 animals express H-2^d restricted $(EYA)_5$ specific clones, the radioresistant H-2^{bxd} thymic elements, responsible for the positive selection of self Ia restricted antigen specific T helper cells [30,31], must be appropriate and sufficient for the selection of such specificities. Therefore, should responder H-2^{bxd} radiosensitive bone marrow derived cells, absent in the H-2^b->F1 animals, be required for the positive selection of H-2^d restricted K3K specific clones [32,33] it would imply that very fine differences in the antigen specificity of maturing T helper cells dictate the employment of diverse positive selection strategies.

Thirdly, a bone marrow derived H-2^b immunoregulatory cell could be responsible for the down regulation and/or deletion of K3K specific clones in the H-2^b->F1 animals. This trait would either have to be recessive since the H-

2^{bxd} F1 bone marrow derived cells do not delete such clones, or a property of the H-2^b non responder cell which is maintained irrespective of the environment in which it arises.

Finally, K3K specific clones may not be generated from the H-2^b bone marrow stem cell source for positive selection to occur. H-2^b T cells may have some intrinsic defect in their capacity to generate T cell receptor specificities that recognize lysine containing peptides of Poly-18 in the context of responder I-A^d gene products. $(EYA)_5$ specific clones derived from Poly-18 primed BALB/c mice use different dominant TcR Vα and Vβ genes than $(EYA)_5$ specific clones derived from Poly-18 primed H-2^b->F1 animals (P. Kilgannon et al. manuscript submitted and unpublished data). Such genotype specific preferences in TcR gene usage may contribute to repertoire diversity restrictions. Thus, underlying the usual limitations imposed upon the developing T cell repertoire by the environment may be a genetically imposed restriction on the diversity of that repertoire.

Table 3.1. Poly - 18 specific T cell clonotypes in BALB/c (H - 2^d) mice.

Antigen (10µM)	Poly - 18 specific T cell hybridoma responses.[a] (CPM x 10^{-3})		
	TYPE A[b] K3K specific	TYPE B[c] $(EYA)_5$ specific	TYPE B[d] $(EYA)_5$ and K3K specific
$(EYA)_5$	1 - 3	> 50	> 50
$EYK(EYA)_4$	> 50	> 50	> 50
$(EYA)_4EYK$	1 - 3	10 - 20	10 - 20
$EYK(EYA)_3EYK$	> 50	1 - 3	> 50

a. T cell hybridoma responses were measured as in Materials and Methods using BALB/c H-2^d spleen cells as APC.
b. Responses of a representative Type A hybridoma A.1.1(16).
c. Responses of a representative Type B hybridoma B15(Chapter 4).
d. Responses of a representative Type B hybridoma B16(Chapter 4).

Table 3.2. Clonotypic analysis of Poly -18 - specific T cells from normal and chimeric mice.[a]

Mice	T Cell Lines	T Cell MHC	APC in vitro	Fusion Partner
Normal				
BALB/c	BALB Line	d	d	BW5147
BALB/c x C57BL/6(F_1)	F_1 Line	bxd	bxd	BW5147
Chimeras				
F_1 --> F_1	F_1 --> F_1 Line	bxd	bxd	BW5147
B6 --> F_1	b --> F_1 Line I	b	bxd	BW5147
B6 --> F_1	b --> F_1 Line II	b	bxd	BW5147
B6 --> F_1	b --> F_1 Line III	b	bxd	BW $\alpha^-\beta^-$

a. For the generation of these T cell lines see Materials and Methods.

Table 3.3. Antigen specific proliferation of Poly - 18 specific T cell lines.

		$[^3H]$ - Thymidine incorporation [a] (CPM x 10^{-3})		
		Challenge Antigen + APC [c]		
T cell lines [b]	T cells/well	No Ag + H-2^bAPC	No Ag + H-2^dAPC	Poly-18 + H-2^dAPC
BALB Line	$5x10^3$	420 +/- 38	1363 +/- 191	62033 +/- 2871
	10^4	1380 +/- 44	6756 +/- 378	95751 +/- 1803
F_1 Line	$5x10^3$	150 +/- 48	7302 +/- 920	36080 +/- 548
	10^4	142 +/- 14	28876 +/- 242	90849 +/- 2081
b --> F_1 Line I	$5x10^3$	N D	1617 +/- 749	43629 +/- 8863
	10^4	438 +/- 10	9881 +/- 125	106578 +/- 4474
b --> F_1 Line II	10^4	474 +/- 52	2780 +/- 186	65680 +/- 3322
b --> F_1 Line III	$5x10^3$	2176 +/- 6	920 +/- 88	36012 +/- 1546
	10^4	4349 +/- 155	3531 +/- 537	66597 +/- 2005
F_1 --> F_1 Line	$5x10^3$	91 +/- 21	1186 +/- 38	60503 +/- 871
	10^4	211 +/- 51	8678 +/- 1980	129156 +/- 4832

a. Proliferation of these T cell lines was measured as described in Materials and Methods. Results represent the mean of duplicate cultures +/- S.D.

b. T cell lines from Table 3.2.

c. C57BL/6 (H-2^b) or BALB/c (H-2^d) spleen cells were used as APC.

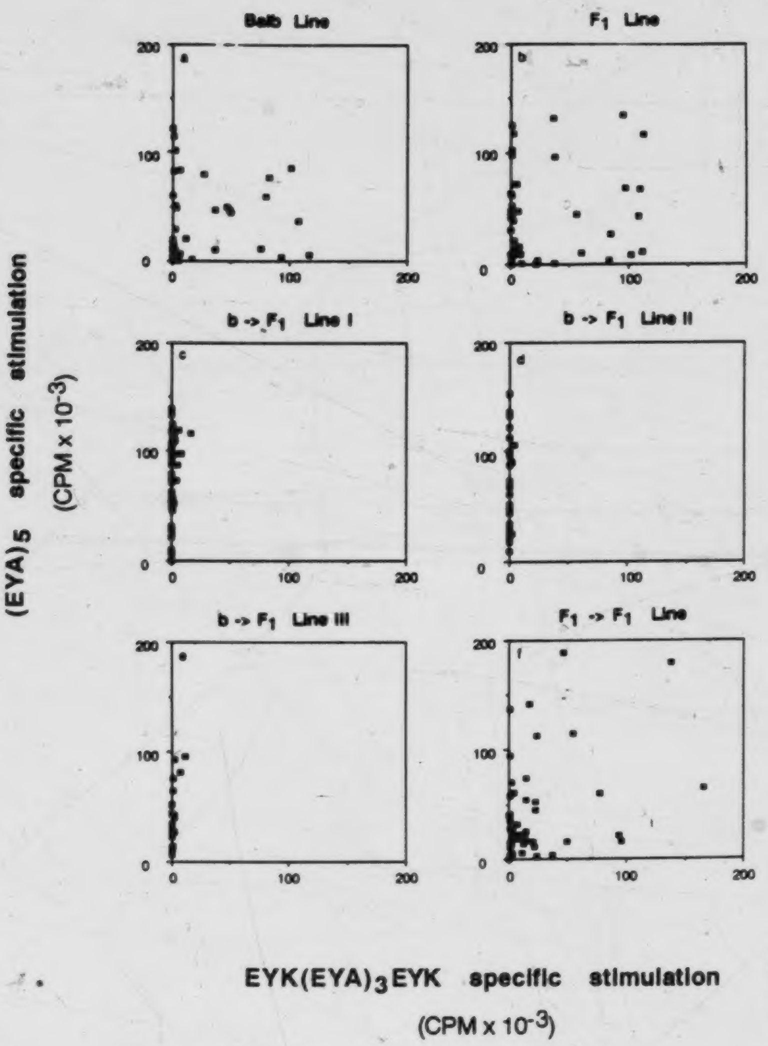

Figure 3.1. Relative frequency of $(EYA)_5$ specific and K3K specific T cell clones in various Poly -18 responder animals. The antigen specificity of T cell hybridomas using the antigens $EYK(EYA)_3EYK$ and $(EYA)_5$ was tested as described in Materials and Methods. CTLL medium control backgrouds were identical to hybridoma plus APC medium control backgrounds, ranging from 1000-3000 CPM for (a,b,c,e,f) and 6000-8000 CPM for (d). Medium control backgrounds were subtracted from antigen specific stimulations which were then plotted in an $(EYA)_5$ specific stimulation (ordinate) versus $EYK(EYA)_3EYK$ specific stimulation (abscisa) matrix. The number of linbro wells tested for each T cell line was (a) 48 in the BALB Line, (b) 45 in the F1 Line, (c) 46 in the b->F1 Line I, (d) 24 in the b->F1 Line II, (e) 20 in the b->F1 Line III and (f) 45 in the F1->F1 Line. Identical results were obtained with another set of BALB/c and F1 derived T cell lines as well as a replicate fusion of line b->F1 Line I.

Table 3.4

Antigen specificity analysis of Poly-18 specific

T cell hybridomas derived from the chimeric line b --> F_1 Line I.

T cell hybridomas [a]	Antigen APC [c]	NONE H-2^d	Poly-18 H-2^d	EYK(EYA)$_3$EYK H-2^d	(EYA)$_5$ H-2^d	NONE H-2^b	Poly-18 H-2^b
		T cell hybridoma responses [b] (CPM x 10^{-3})					
Ch 3 - B - 3 - 2		1	101	1	46	76	103
Ch 3 - B - 9 - 4		1	84	1	24	4	4
Ch 3 - B - 13 - 6		1	136	1	85	112	107
Ch 3 - B - 14 - 3		1	34	1	104	122	115
Ch 3 - B - 20 - 2		1	56	1	11	1	1
Ch 3 - D - 4 - 8		1	109	1	37	1	1
Ch 3 - D - 7 - 1		1	43	1	24	10	13
Ch 3 - D - 17 - 8		1	41	1	36	12	13
Ch 3 - F - 20 - 4		1	60	3	47	1	1
Ch 3 - F - 23 - 4		1	46	3	48	2	2
Ch 3 - F - 25 - 2		1	43	1	41	1	1
Ch 3 - F - 29 - 4		1	35	1	18	1	1
Ch 4 - 11 - 5		1	71	1	65	2	3

a. T cell hybridomas were subcloned by limiting dilution.
b. T cell hybridoma responses were measured as in Materials & Methods.
c. C57BL/6 (H-2^b) or BALB/c (H-2^d) spleen cells were used as APC.

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CHAPTER 4

The Poly-18 epitope $(EYA)_5$ is the focus of a highly polyclonal T cell response

(Paper III)

Introduction

The immune system utilizes a diverse T cell repertoire for the recognition of foreign antigens in the context of self major histocompatibility complex (MHC) gene products [1]. T helper cells recognize antigens in the context of self Class II MHC (Ia)molecules [2-4]. Large protein antigens are usually degraded, or processed , by antigen presenting cells (APC) into smaller peptides [5]. Some of these peptides then associate with self Ia molecules on the surface of the APC and are recognized by T cells bearing the appropriate T cell receptors (TcR). Most TcR's are clonally expressed as alpha and beta chain heterodimers in association with the CD3 complex on the cell surfaces of T cells [6]. The number of potential TcR's that can be generated as a result of combinatorial permutations of variable(V) genes of alpha and beta chains which are imprecisely joined and N-region diversified to joining(J) gene segments has been estimated at 10^7 [7]. This TcR diversity is matched by the diversity of immunogenic epitopes on foreign antigens. Large protein molecules, such as insulin [8], staphylococcal nuclease [9], influenza hemagglutinin [10], cytochrome c [11], myoglobin [12] and ovalbumin [13], can be processed into small peptide fragments representing distinct T cell epitopes some of which are immunodominant and elicit vigorous T cell responses. In addition, small peptide fragments representing immunodominant epitopes, such as the sperm whale myoglobin fragments 110-121 and 106-118 [14-16] or the hen egg lysozyme fragments 46-61 and 34-45 [17,18] or 74-86 and 81-96 [19,20], elicit heterogeneous T cell responses although they do not require any further antigen processing . A single immunodominant epitope can thus appear to be a compilation of several overlapping epitopes confined to a small region within a larger protein molecule.

We examined the potential diversity of the T cell response directed to one of the immunodominant epitopes of Poly-18, Poly EYK(EYA)$_5$, a synthetic alpha-helical polypeptide [21]. Responsiveness to Poly-18 is under Ir gene control. Mice of the H-2^d haplotype ie. BALB/c, DBA/2J, B10.D2 and C3H.OH are responders and elicit Poly-18 specific antibodies, DTH and proliferating T cells while mice of the H-2^b haplotype ie. C57BL/6, C57BL/10 and C3H/SwSn or H-2^k haplotype ie. CBA/J, CBA/CaJ, B10.Br, C3H.A and C3H/HeJ are Poly-18 nonresponders [22]. Poly-18 has two immunodominant epitopes represented by the minimum peptides EYK(EYA)$_4$ and (EYA)$_5$. Two major Poly-18 specific (I-A^d restricted) T cell clonotypes, Type A and Type B, are directed at these two immunodominant epitopes in BALB/c (H-2^d) mice [23]. Type A clones are absolutely dependent on the presence of N-terminal lysine in position 3 and respond to the Poly-18 derived peptide EYK(EYA)$_4$ while Type B clones are lysine independent and respond to the Poly-18 derived peptide (EYA)$_5$. In addition these minimum Poly-18 sequences have been shown not to require further antigen processing prior to presentation to the Poly-18 specific T cell hybridomas [24].

Although the (EYA)$_5$ epitope appears to be relatively simple in terms of its amino acid sequence, consisting of a 3 amino acid repeat, it does represent one of the Poly-18 immunodominant epitopes. We therefore asked whether a simple epitope such as (EYA)$_5$ can serve as the focus of a polyclonal T cell response. We proceeded to answer this question by analyzing the fine antigen specificity of 21 randomly chosen Type B, $(EYA)_5$ specific, T cell hybridomas derived from H-2^d, H-2^{bxd} and H-2^b->H-2^{bxd} mice (Chapter 3). We used a panel of alanine and/or lysine substituted $(EYA)_5$ peptides to analyze the peptide specific responses and a panel of haplotype variant splenocytes in the absence of exogenous peptide antigens to analyze the allo crossreactivity patterns of the 21 Type B T cell hybridomas in order to determine the number of unique antigen reactivity patterns represented by this T cell population. We find that 13 of the 21 Type B T cell hybridomas have a distinct fine antigen specificity although all of them are specific for the $(EYA)_5$ epitope. The $(EYA)_5$ epitope thus appears to be the focus of an impressively heterogeneous T cell response.

Materials and Methods

Mice- C57BL/6(H-2^b), CBA/J(H-2^k), DBA/1J(H-2^q), B10.RIII(71NS)/SN (H-2^r), B10.M/SN(H-2^f), SJL/J(H-2^s) and (BALB/c x C57Bl/6)F1 (H-2^{bxd}) mice were obtained from The Jackson Laboratory, Bar Harbor, ME. BALB/c(H-2^d) mice were bred at the Ellerslie Animal Farm of the University of Alberta, Canada. B10.GD(H-2^d) mice were bred in our colony from breeding stock provided by Dr. C. David, Mayo Clinic, Rochester, Minn. Female mice were used at 2-6 mo of age.

Peptides- Poly-18 and the various related peptides were prepared by the Merrifield solid phase peptide synthesis technique [25] as described previously [21,26]. For functional assays, peptides were dissolved in saline, pH adjusted to 7.2 with 0.1N NaOH, sterilized by filtration through a 0.22 Um filter and stored at -20'C. Amino acid abbreviations: K; lysine, E; glutamic acid, Y; tyrosine, A;alanine.

Bone marrow radiation chimeras- (BALB/c x C57BL/6)F1 mice were irradiated with 1000 R and reconstituted 24 hrs later with an intravenous injection of anti-Thy 1.2 plus complement treated 10^7 (BALB/c x C57BL/6)F1 or C57BL/6 bone marrow(BM) cells. 3-6 months later the chimerism was analyzed using a Glucose phosphate isomerase (GPI) assay [27] which measures the presence of strain specific isozymes. All chimeric animals survived and had no signs of graft versus host disease (GVHD). All were 100% donor in their lymph nodes and peripheral red blood cells 3 months after bone marrow transplantation and were used for antigen priming 4-6 months post transplantation.

T cell lines- A series of BALB/c, 5 (BALB/c x C57BL/6)F1 and 10 C57BL/6->(BALB/c x C57BL/6)F1 mice mice were primed in both hind footpads with

30μg of Poly-18 in complete freunds adjuvant(CFA). 7-9 days later lymph node T cells were nylon wool separated and 2-4x10^5 T cells/ml were cultured with 3x10^6 irradiated(4000 R) spleen cells/ml and 10μg Poly-18/ml in RPMI 1640 (Gibco) containing 10% fetal bovine serum(FCS), 5x10^{-5} M 2-mercaptoethanol, 10 mM Hepes, 2 mM glutamine and penicillin-streptomycin. Poly-18 specific T cell lines of BALB/c, (BALB/c x C57BL/6)F1 and C57BL/6->(BALB/c x C57BL/6)F1 origin were carried on BALB/c, (BALB/c x C57BL/6)F1 and (BALB/c x C57BL/6)F1 irradiated spleen cells, respectively, for 1-4 months prior to fusing with BW5147 thymoma cells.

T cell fusions- T cell fusions were performed as previously described using the standard AKR thymoma line BW5147 and a standard PEG fusion protocol [23]. Fusion mix was plated in 24 well linbro plates at 2x10^5 BW5147 cells/2ml/well. T cell hybridomas were initially grown in Hypoxanthine-Thymidine medium for 2 weeks and eventually adapted to drug free medium containing only RPMI 1640 and 10% FCS. Hybridomas were then tested for IL-2 release in the presence of antigen (Poly-18 and the peptides (EYA)$_5$ and EYK(EYA)$_3$EYK) and BALB/c APC. Selected clones were subcloned at 0.1 cell/well one to three times as deemed necessary for obtaining a clonal population. T cell hybridomas of BALB/c origin came from 5 separate fusions (i-v). Hybridomas B1,B4,B5 and B8 from fusion(i), B6 from fusion(ii), B7 and B9 from fusion(iii), B15 from fusion(iv) and B16 and B17 from fusion(v).

IL-2 release assay- Antigen specificity tests were performed by coculturing T hybridoma cells (10^5) with (10^6) irradiated spleen cells or (5x10^4) TA3 cells [28] as APC and various doses of antigen in 300μl RPMI 1640 and 10%FCS for 24 hrs. The supernatants were diluted at 1:2 or 1:8 in 200 μl and tested for the presence of IL-2 by their ability to support the growth of (10^4) IL-2 dependent CTLL cells, as measured by ^{3}H-thymidine incorporation. Supernatants were always diluted at 1:2 when spleen cells were used as APC and at 1:8 when TA3, I-A$^{d/k}$ I-E$^{d/k}$ bearing B cell hybridoma, cells were used as APC. TA3 cells were kindly provided by Dr. L. Glimcher, Harvard Laboratory of Public Health, Boston and CTLL cells by Dr. T. Delovitch, University of Toronto, Canada.

Results

Generation of Poly-18 reactive Type B (I-A^d restricted, $(EYA)_5$ specific) T cell hybridomas from H-2^d,H-2^{bxd} and H-2^b->H-2^{bxd} mice.

Responsiveness to Poly-18 is under Ir-gene control. Mice of the H-2^d haplotype ie. BALB/c are responders and mice of the H-2^b haplotype ie. C57BL/6 are nonresponders. Responsiveness is dominant over nonresponsiveness and heterozygous H-2^{bxd} mice ie. (BALB/c x C57BL/6)F1 are also Poly-18 responders [22]. In addition, Poly-18 nonresponder C57BL/6 (H-2^b) bone marrow cells contain T cell precursors which can be educated in an irradiated (BALB/c x C57BL/6)F1 (H-2^{bxd}) responder environment to recognize Poly-18 in the context of the responder H-2^d MHC gene products (Chapter 3). Poly-18 specific T cells from the lymph nodes of normal BALB/c (H-2^d) and (BALB/c x C57BL/6)F1 (H-2^{bxd}) as well as bone marrow radiation chimeric C57BL/6->F1 (H-2b->H-2^{bxd}) mice were used to generate T cell hybridomas. Ten Type B T cell hybridomas from BALB/c, 3 from (BALB/c x C57BL/6)F1 and 8 from C57BL/6->F1 mice were selected on the basis of their reactivity to the Poly-18 derived peptide $(EYA)_5$ in the context of H-2^d bearing splenic antigen presenting cells (APC). (Table 4.1) Analysis of Type B T cell hybridomas derived from the three different responder mouse populations is useful in assessing whether environmental factors influence either the heterogeneity or the fine antigen specificity profiles of these Poly-18 specific T cell clones.

Previous studies revealed that the Poly-18 specific response in H-2^d strains is restricted to I-A^d [23]. Activation of Poly-18 specific T cell hybridomas was shown to be specifically inhibited by the anti-I-A^d monoclonal antibody

(MAb) MKD6 but not the anti-I-A^k MAb 10.3.6. Furthermore, only APC bearing I-A^d molecules could stimulate the Poly-18 specific T cell hybridomas. We demonstrate that our Type B T cell hybridomas, as representatives of the Poly-18 specific T cell response, are also I-A^d restricted. B10.GD (I-A^d, I-E^o) spleen cells, which do not express the I-E gene product, are just as effective as BALB/c (I-A^d, I-E^d) spleen cells in presenting Poly-18 to our Type B T cell hybridomas. (Table 4.2)

Fine antigen specificity analysis of Type B T cell hybridomas.

A panel of synthetic peptides, consisting of $(EYA)_5$, $(EYA)_5$ substituted with alanine residues at position 5, 7 or 8 and $EYK(EYA)_4$ substituted with alanine residues at position 5, 7 or 10 were used to analyze the fine antigen specificity of our Type B T cell hybridomas. (Table 4.3) Our choice of peptides was based on an extensive fine antigen specificity analysis of the Type B and Type A Poly-18 specific T cell hybridomas B.1.1(B15) and A.1.1, respectively, which indicated that the amino acid residues at positions 7, 8 and 10 are likely to be involved in critical interactions involving the TcR and or Ia molecules while the amino acid residue at positon 5 did not appear to be a critical residue for the activation of these two T cell hybridomas ([29] and M. Boyer et al. manuscript in preparation). We used $(EYA)_5$ as well as $EYK(EYA)_4$ substituted peptides since Type B clones, although not dependent on the presence of lysine, do respond to $(EYA)_5$ substituted with lysine residues at position 3 ie.$EYK(EYA)_4$ or position 15 ie. $(EYA)_4EYK$ [24]. However, only Type A [24] and a subpopulation of Type B clones (shown in Tables 4.4 and 4.5) respond strongly to the peptide $EYK(EYA)_3EYK$ which contains lysine residues at both positions 3 and 15.

The B cell hybridoma TA3 ($I\text{-}A^{d/k}$: $I\text{-}E^{d/k}$) was used as APC in all instances for the presentation of peptide antigens to our T cell hybridomas since we have shown previously that Poly-18 and its fragments do not require antigen processing prior to being presented to Poly-18 specific T cell hybridomas [24]. TA3 cells also have high levels of cell surface Ia which enables them to present peptide antigens more effectively than splenic APC in vitro [28]. All of the Type B T cell hybridomas were tested at low (10μM) as well as high (100μM) antigen concentrations. This gave us the option of using antigen dose dependent differences in the activation of the T cell hybridomas as a criterion of defining their fine antigen specificities. However, the T cell hybridoma population turned out to be very heterogeneous and consequently only responses obtained at one antigen dose, the 100μM concentration, were necessary to consider in defining the various antigen reactivity patterns.

As an extension of the fine antigen specificity analysis using peptide antigens we also used a series of haplotype variant splenocytes to determine the allo crossreactivity patterns of our Type B T cell hybridomas. Spleen cells from C57BL/6($H\text{-}2^b$), CBA/J($H\text{-}2^k$), DBA/1J($H\text{-}2^q$), B10.M/SN($H\text{-}2^f$), B10.RIII(71NS)/SN($H\text{-}2^r$) and SJL/J($H\text{-}2^s$) mice were used to stimulate the T cell hybridomas in the absence of any exogenous peptide antigens.

Type B ($I\text{-}A^d$ restricted, $(EYA)_5$ specific) T cell hybridomas represent a diverse spectrum of antigen reactivity patterns.

Using the antigen panel, consisting of the alanine substituted $(EYA)_5$ and $EYK(EYA)_4$ peptides shown in Table 4.3, we can discern 6 distinct antigen reactivity patterns among the 10 BALB/c derived Type B T cell hybridomas.(Table 4.4) Hybridomas B4, B5, B6, B15 and B17 each have a unique fine antigen specificity while hybridomas B1, B7, B8, B16 and B9 appear to have similar antigen specificities. However, hybridoma B9 can be separated from hybridomas B1, B7, B8 and B16 on the basis of its allo crossreactivity on CBA/J(H-2^k) spleen cells.(Table 4.6) The BALB/c derived Type B T cell hybridomas thus represent a total of 7 unique antigen reactivity patterns.

The 3 (BALB/c x C57BL/6)F1 derived Type B T cell hybridomas F1, F3 and F4 each have a unique antigen reactivity pattern as determined by their responses to our panel of alanine substituted $(EYA)_5$ and $EYK(EYA)_4$ peptides.(Table 4.5) These 3 antigen reactivity patterns are distinct from the 7 antigen reactivity patterns represented by the BALB/c derived Type B T cell hybridomas.

The 8 C57Bl/6->F1 chimera derived Type B T cell hybridomas can be separated into 4 distinct antigen reactivity patterns based on their responses to our panel of alanine substituted $(EYA)_5$ and $EYK(EYA)_4$ peptides. (Table 4.5) Hybridomas Ch3, Ch7, Ch9 and Ch10 have a similar antigen reactivity pattern. In addition, analysis of the allo crossreactivity patterns of these 4 hybridomas indicates that all 4 of them crossreact on allogeneic CBA/J(H-2^k) spleen cells and all except hybridoma Ch3 also crossreact on C57Bl/6(H-2^b) spleen cells.(Table 4.6) Hybridomas Ch4 and Ch8 also have a similar antigen reactivity pattern. This pattern overlaps with that of the BALB/c derived hybridoma B6. The remaining two hybridomas, Ch2 and Ch11, each have a unique antigen specificity. The antigen reactivity pattern of hybridoma Ch2 appears similar to that of the BALB/c derived hybridomas B1, B7, B8 and B16 while the antigen reactivity pattern of hybridoma Ch11 appears similar to that of the (BALB/c x C57BL/6)F1 derived hybridoma F3. However, hybridomas Ch2 and Ch11 represent unique antigen specificities based on their allo crossreactivities on B10.M/SN(H-2^f) and DBA/1J(H-2^q) spleen cells, respectively.(Table 4.6) The

C57BL/6->F1 chimera derived Type B T cell hybridomas thus introduce another 3 unique antigen reactivity patterns to the 7 and 3 already identified among the BALB/c and (BALB/c x C57Bl/6)F1 derived Type B T cell hybridomas, respectively. In total, we can discern 13 unique antigen reactivity patterns among the 21 Type B, I-A^d restricted, $(EYA)_5$ specific, T cell hybridomas analyzed.(Table 4.7)

Type B hybridomas derived from chimeric C57BL/6->F1 mice are less heterogeneous than those derived from normal BALB/c mice.

We use the frequency of unique antigen reactivity patterns in a given T cell population as an indicator of the T cell heterogeneity in that population. We find that 6/10 of the Type B T cell hybridomas derived from BALB/c mice ie. B4, B5, B6, B9, B15 and B17 represent their own unique antigen reactivity patterns while only 2/8 of the Type B T cell hybridomas derived from C57BL/6->F1 chimeric mice ie. Ch2 and Ch11 represent their own unique antigen reactivity patterns. The rest of the BALB/c derived Type B hybridomas ie. B1, B7, B8 and B16 fall into a single antigen reactivity pattern and the rest of the C57BL/6->F1 chimeric derived Type B hybridomas fall into two antigen reactivity patterns, one of which is represented by hybridomas Ch4 and Ch8 and the other by hybridomas Ch3, Ch7, Ch9 and Ch10. In total, 70% (7/10) of the BALB/c derived Type B hybridomas represent unique antigen reactivity patterns compared to 50% (4/8) of the C57BL/6->F1 chimeric derived Type B hybridomas . (Table 4.7)

Comparison of the $(EYA)_5$ specific T cell repertoires in BALB/c and C57BL/6->F1 chimeric Poly-18 responder animals.

The $(EYA)_5$ specific antigen reactivity patterns represented by the Type B T cell hybridomas derived from the various Poly-18 primed responder animals are very diverse and essentially nonoverlapping. However, we can discern similarities as well as differences when comparing the overall $(EYA)_5$ specific T cell responses between the BALB/c and C57BL/6->F1 chimeric animals. The $(EYA)_5$ specific T cell repertoires of BALB/c and C57BL/6->F1 chimeric animals are similar in two respects.

Firstly, CBA/J(H-2^k) allocrossreactivity is a major component of the Type B, $(EYA)_5$ specific, T cell response in both BALB/c and C57BL/6->F1 chimeric animals. Five of the BALB/c derived Type B hybridomas ie. B4, B5, B9, B15 and B17 and four of the C57BL/6->F1 chimera derived Type B hybridomas ie. Ch3, Ch7, Ch9 and Ch10 crossreact on allogeneic CBA/J(H-2^k) spleen cells. CBA/J(H-2^k) allocrossreactivity is thus the property of 50% of the BALB/c and C57BL/6->F1 chimera derived Type B T cell hybridomas. (Table 4.7)

Secondly, the frequency of strongly responding Type B T cell hybridomas to most of the alanine substituted $(EYA)_5$ or $EYK(EYA)_4$ peptides is similar in the BALB/c and C57BL/6->F1 chimeric animals. The frequency of BALB/c and C57BL/6->F1 chimera derived Type B hybridomas responding to the peptide $(EYA)_5$sub5 is 60% and 50%, to $(EYA)_5$sub7 is 80% and 100%, to $(EYA)_5$sub8 is 0% and 12%, to $EYK(EYA)_4$sub5 is 70% and 50% and to $EYK(EYA)_4$sub10 is 80% and 100%, respectively. (Table 4.8) Thus, anywhere between 50 and 100 % of the BALB/c or C57BL/6->F1 chimera derived Type B T cell hybridomas respond to the peptides $(EYA)_5$sub5, $(EYA)_5$sub7, $EYK(EYA)_4$sub5 and $EYK(EYA)_4$sub10 while the response to peptide $(EYA)_5$sub8 is severely under represented in both BALB/c and C57BL/6->F1 chimeric animals.

We note one difference between the $(EYA)_5$ specific T cell repertoires of BALB/c and C57BL/6->F1 chimeric animals based on our previous analysis of BALB/c and C57BL/6->F1 chimera derived Poly-18 specific T cells which revealed differences between the two responding T cell populations based on their ability to respond to the lysine containing peptide K3K(Chapter 3). We observed that BALB/c derived Poly-18 specific T cell populations contain approximately twice as many weakly as strongly K3K reactive T cell clones while C57BL/6->F1 chimera derived Poly-18 specific T cells are almost exclusively of the weak K3K reactive type. Concordingly, several of the BALB/c derived Type B hybridomas are representative of strongly K3K reactive clones ie. B7, B8, B9, B16 and B17 and respond to low (10μM) concentrations of the K3K peptide while others represent weakly K3K reactive clones ie B1, B4, B5, B6 and B15 and respond only to high (100μM) concentrations of the K3K peptide or do not respond at all. (Table 4.5) As expected, only one of the C57BL/6->F1 chimera derived Type B hybridomas ie. Ch11 represents a strongly K3K reactive clone while the rest ie. Ch2, Ch3, Ch4,Ch7, Ch8, Ch9, andCh10 all represent weakly K3K reactive clones. (Table 4.6)

Discussion

Our fine antigen specificity analysis of 21 Type B (I-A^d restricted, (EYA)$_5$ specific) T cell hybridomas derived from three Poly-18 primed responder mouse populations indicates that the (EYA)$_5$ epitope is the focus of a very heterogeneous T cell response. The 21 Type B T cell hybridomas represent at least 13 unique antigen reactivity patterns. This heterogeneity is also reflected in the TcR Vα and Vβ gene usage of these hybridomas (P. Kilgannon et al. submitted for publication and unpublished). At least 15 of these 21 Type B T cell hybridomas use unique TcR VαVβ gene pair combinations. Although the Poly-18 specific T cell respose is dominated by one Vα(Vα11) and one Vβ(Vβ6) gene, it is very diverse because the dominant TcR V region bearing chains associate with a variety of partner chains and not with each other.

The 10 BALB/c derived Type B T cell hybridomas analyzed represent at least 7 unique antigen reactivity patterns and use at least 7 unique VαVβ gene pair combinations. The 4 Type B hybridomas B1, B7, B8 and B16, have a similar antigen reactivity pattern but each uses a distinct pair of TcR VαVb genes. In addition there appears to be no specific preference for any Vα or Vβ gene used among these 4 hybridomas. It may be that certain TcR VαVβ gene combinations result in the formation of similar antigen combining sites. Alternatively, a more extensive panel of substituted (EYA)$_5$ peptides may be able to distinguish between these apparently similar clones. Of the 6 BALB/c derived Type B clones possessing unique antigen reactivity patterns, hybridomas B4, B5, B15 and B17 have distinct TcR's while hybridomas B6 and B9 use the same VαVβ gene combination. Analysis of Jα and Jβ genes of hybridomas B6 and B9 may reveal the basis for their variable antigen reactivity patterns. The use of an alternate Jα region in two cytochrome c specific T cell clones [30,31] or alternate Jα and Jβ regions in two myelin basic protein specific T cell clones [32] has been shown to result in subtle antigen specificity differences between them.

The 3 (BALB/c x C57BL/6)F1 derived Type B hybridomas F1, F3 and F4, each have a unique antigen reactivity pattern and each uses a distinct pair of TcR VαVβ genes. In addition, the 3 antigen reactivity patterns observed in the (BALB/c x C57BL/6)F1 T cell population are distinct from those observed in the BALB/c T cell population.

The C57BL/6->F1chimera derived Type B T cell hybridomas are much less heterogeneous than the BALB/c derived Type B T cell hybridomas. The 4 C57BL/6->F1chimera derived Type B hybridomas Ch3, Ch7, Ch9 and Ch10, have a similar antigen reactivity pattern and also use the same TcR VαVβ gene pair combination. Although hybridoma Ch3 is the only one of the four hybridomas which does not crossreact on C57Bl/6(H-2^b) spleen cells, it is also the only one which has deleted its BW5147 Vα1 chain. These two events are possibly related since the BW5147 Vα1 chain has been implicated in imparting H-2^b reactivity in some T cell hybridomas [33]. Hybridomas Ch4 and Ch8 also have a similar antigen reactivity pattern. This pattern overlaps with that of the BALB/c derived hybridoma B6, but in this case all three hybridomas use a different TcR VαVβ gene pair. The remaining two hybridomas Ch2 and Ch11 each have a unique antigen specificity and a distinct TcR.

There does not appear to be any correlation between a given antigen reactivity pattern and a TcR Vα and/or Vβ gene being used. The only correlation observed involves the dominant Vβ gene, Vβ6. Eventhough Vβ6 associates with a variety of Vα chains to produce several unique TcR VαVβ gene pair combinations, 8 of the 10 Type B T cell hybridomas using the dominant Vβ6 gene ie.B4, B5, B17, F4, Ch3, Ch7, Ch9 and Ch10, crossreact on allogeneic CBA/J(H-2^k) spleen cells. These responses are likely to involve the recognition of Mls-1^a since these hybridomas do not crossreact on TA3(H-2^{kxd}) cells and Poly-18 specific T cell lines derived from BALB/c mice proliferate in response to CBA/J(H-2^k,Mls-1^a,Mls-2^a) and AKR(H-2^k,Mls-1^a,Mls-2^b) but not B10.BR(H-2^k,Mls-1^b,Mls-2^b) spleen cells(data not shown). A correlation involving Mls-1^a reactivity and the TcR genes Vβ6 and Vβ8 has been described previously in Mls-1^a bearing mice [34,35].

Polyclonal T cell responses directed at immunodominant epitopes have been observed in several antigen systems. Antigen specific T cell clones or T cell hybridomas have been analyzed for fine antigen specificity using proteolytic fragments as well as related synthetic peptides of various protein antigens. Haplotype variant spleen cells have also been used for the presentation of antigens or in the absence of antigen to determine allocrossreactivity patterns. In cytochrome c, at least 5 phenotypically distinct groups of T cell clones respond to the immunodominant epitope 81-103 in the context of I-E^k MHC molecules [31]. Sperm whale myoglobin (SWM) contains several immunodominant epitopes which elicit heterogeneous T cell responses. At least 3 different I-E^d restricted and 3 different I-A^d restricted T cell clones respond to the 11 amino acid long SWM epitope 110-121 and the 12 amino acid long SWM epitope 106-118, respectively [14]. Similarly, hen egg lysozyme (HEL) contains several immunodominant epitopes. The use of B cell hybridomas bearing mutated I-A^k alpha or beta chains for the presentation of HEL related peptides to HEL specific T cell hybridomas revealed that the epitope 46-61 elicits at least 4 and the epitope 34-45 at least 2 unique I-A^k restricted HEL specific T cell clones [17]. In addition, the HEL epitopes 74-86 and 81-96 have been shown to elicit at least 3 distinct I-A^k and 3 distinct I-A^b restricted HEL specific T cell clones, respectively [20]. Even haptens can elicit heterogeneous T cell responses. The hapten p-Azobenzenearsonate elicits at least 3 different T cell specificities in A/J mice [36]. Although the $(EYA)_5$ epitope may appear to elicit a more heterogeneous T cell response than some of the immunodominant epitopes just mentioned, a more extensive antigen specificity analysis, such as was performed in the HEL system and in our system, may be necessary in order to reveal a greater level of response diversity in the other antigen systems.

The use of dominant TcR Vα or Vβ genes, which has been observed in several antigen systems, is probably not indicative of a decreased potential for the generation of heterogeneous T cell responses although it may reflect the preferential selection of some TcR chains in the thymus. The T cell responses elicited by cytochrome c, p-Azobenzenearsonate and SWM are phenotypically quite heterogeneous even though Vα11 is used by the majority of cytochrome c specific T cells [30,37], Vα3 by most arsonate specific T cells [38] and Vβ8 by almost all I-E^d but not I-A^d restricted SWM specific T cells [14]. Similarly, the dominant use of Vβ6 or Vα11 by Poly-18 specific T cells does not appear to limit the diversity of the Poly-18 specific T cell repertoire. Although the dominant TcR V gene usage correlates with general Poly-18 reactivity it does not correlate with any particular Poly-18 specific T cell phenotype. Unlike the use of dominant TcR Vα or Vβ genes, the use of a dominant TcR VαVβ gene pair is potentially indicative of a decreased T cell response heterogeneity. The immunodominant epitope of myelin basic protein (MBP), an N-terminal nonapeptide, elicits one major T cell phenotype represented by 2 groups of T cells bearing identical TcR VαVβ gene pairs but different Jβ segments [32]. However, even under these limiting conditions two minor T cell phenotypes are also elicited in response to MBP. One minor phenotype does not use the dominant Vβ gene while the other uses the dominant VαVβ gene pair but alternate Jα and Jβ gene segments. Thus, even an antigen system using a dominant TcR VαVβ gene pair has the capacity to generate some T cell response heterogeneity. This implies that antigen systems which are not limited to using a dominant TcR VαVβ gene pair will almost invariably generate a heterogeneous T cell response.

Although immunodominant epitopes are the focus of heterogeneous T cell responses the rest of the molecules containing these epitopes are apparently not recognized by the available T cell repertoire. Since the responder status of different strains of mice to some protein antigens is dictated by MHC-linked immune response (Ir) genes, several mechanisms have been proposed to explain the role of these genes on immune responsiveness [39]. First, since T cells recognize antigen only in the context of self MHC molecules, some peptides may not have the ability to bind to self Ia molecules and will thus not be recognized by available T cells. This determinant selection mechanism has been supported by observations that various immunodominant peptides can bind directly to Ia molecules, obtained from responder mouse strains, which are capable of presenting the peptides but not to Ia molecules, obtained from nonresponder mouse strains, which do not present the peptides [40,41]. Second, intrathymic deletion of maturing T cell clones that are reactive to self determinants, which is necessary for the maintenance of self tolerance, may lead to functional holes in the expressed T cell repertoire [34,35,42-44]. Third, non responsiveness can be actively maintained by regulatory Ts cells [45-47].

The determinant selection hypothesis can easily explain why only some regions of foreign molecules are immunogenic by proposing that the nonimmunogenic parts do not bind to self Ia molecules. Furthermore, it has been suggested that the heterogeneity of the T cell responses directed at immunodominant epitopes precludes deletion mechanisms since too many different clones would have to be eliminated for any given self epitope which would severely limit the diversity of the expressed T cell repertoire. However, one important feature of the heterogeneous T cell responses directed at immunodominant epitopes which makes the deletion mechanism a viable alternative is the presence of clonotypic hierarchies.

The fine antigen specificity analysis of our 21 Type B T cell hybridomas indicates that the Poly-18 specific T cell repertoire contains very few T cell clones capable of responding to the alanine substituted $(EYA)_5$ peptide, $(EYA)_5$sub8. Only 14% of the Type B T cell hybridomas are capable of responding to the $(EYA)_5$sub8 peptide while 50 to 90% of the Type B T cell hybridomas are capable of responding to the other alanine substituted $(EYA)_5$ and $EYK(EYA)_4$ peptides analyzed. Responses to the $(EYA)_5$sub8 peptide are greatly under represented in the Poly-18 specific T cell repertoire even though in vivo priming studies indicate that the two immunodominant epitopes of Poly-18, $(EYA)_5$ and $EYK(EYA)_4$, are both capable of eliciting $(EYA)_5$sub8 reactive T cell clones(Chapter 2). Similar observations have been made in the HEL antigen system where HEL derived peptides have been shown to elicit a broader range of T cell specificities than the native HEL molecule [48,49]. It thus appears that different forms of the same antigen select for different dominant clonotypes. Similarly, two closely related peptides can select for different dominant clones. The peptide $EYK(EYA)_3EYK$ is immunogenic and elicits Poly-18 reactive T cell clones while the peptide $EYKAAA(EYA)_2EYK$, which only differs from the first peptide by 2 amino acids, is also immunogenic but does not elicit Poly-18 reactive T cell clones(Chapter 2). The functional deletion of one or a few dominant T cell clonotypes may therefore be sufficient to render an epitope relatively nonimmunogenic even though many crossreactive T cell clonotypes, possibly representing dominant T cell clonotypes of other epitopes, remain intact. At least two lines of evidence support this idea.

Firstly, we have observed that Poly-18 Nonresponder C57BL/6 (H-2^b)->Responder (BALB/c x C57BL/6)F1 (H-2^{bxd}) bone marrow radiation chimeras elicit Poly-18 specific T cell responses which are almost completely devoid of strongly K3K reactive T cell clones(Chapter 3). This functional absence of the strongly K3K reactive T cell clones in the C57BL/6->F1 chimeras may be the reason why the $(EYA)_5$ specific T cell repertoire is less heterogeneous in these animals compared to normal BALB/c (H-2^d) animals. However, the entire Poly-18 specific T cell repertoire is not affected in the C57BL/6->F1 chimeras eventhough the Poly-18 specific T cell clones appear to have many similar antigen crossreactivities. Furthermore, the fine antigen specificity analysis of our Type B T cell hybridomas does not reveal any easily identifiable features which are unique to strongly versus weakly K3K reactive T cell clones. Secondly, neonatal tolerance studies using three cytochrome c derived peptides, differing by only one amino acid each, demonstrate that tolerance can be induced to each individual peptide without severely affecting the responses to the other two peptides [50]. It therefore seems likely that only epitopes which are identical to self epitopes and share identical clonal hierarchies would be rendered nonimmunogenic by deletion of the appropriate dominant clonotypes while even very closely related epitopes would be unaffected or only minimally affected.

Table 4.1. Poly - 18 reactive Type B (I-A^d restricted, (EYA)$_5$ specific) T cell hybridomas.[a]

T cell source : BALB/c (H-2^d)

Hybridoma :

B1	(D38-1-6)	B8	(D38-45-3)
B4	(D38-34A-4)	B9	(E-22-2-6)
B5	(D38-34A-6)	B15	(B.1.1)
B6	(K3-8-1-2)	B16	(BB1-19R3r7)
B7	(E-34-1-5)	B17	(AA32b-R3r2c)

T cell source : (BALB/c x C57BL/6)F$_1$ (H-2^{bxd})

Hybridoma :

F1	(F39-43-2)
F3	(F39-41-6)
F4	(F39-25-3)

T cell source : C57BL/6->(BALB/c x C57BL/6)F$_1$ (H-2^b->H-2^{bxd}) bone marrow radiation chimeras

Hybridoma :

Ch2	(Ch3-F-23-4)	Ch8	(Ch3-B-9-1)
Ch3	(Ch3-F-25-2)	Ch9	(Ch3-B-13-6)
Ch4	(Ch3-D-4-8)	Ch10	(Ch3-B-14-3)
Ch7	(Ch3-B-3-2)	Ch11	(Ch3-D-9-10)

a. Mice were immunized with Poly - 18 and T cell lines and hybridomas were generated as described in Materials and Methods.

Table 4.2. Poly-18 reactive, Type B T cell hybridomas are I-A^d restricted.

APC	Antigen	Antigen Dose µM	Type B T cell hybridoma responses (CPM x 10^{-3})									
			B4	B17	B5	B15	B9	B1	B7	B8	B16	B6
BALB/c (I-A^d,I-E^d)	-	-	1	1	1	1	ND	ND	1	1	1	1
	Poly -18	10	45	86	66	53	ND	ND	66	39	83	47
B10.GD(I-A^d,I-E^o)	-	-	1	1	1	1	ND	ND	1	1	1	1
	Poly -18	10	72	96	83	53	ND	ND	61	50	81	56

APC	Antigen	Antigen Dose µM	CH4	Ch8	Ch3	Ch7	Ch9	Ch10	Ch2	Ch11	F3	F4	F1
BALB/c (I-A^d,I-E^d)	-	-	1	1	1	1	1	1	1	1	1	1	1
	Poly -18	10	65	31	52	32	67	54	81	14	98	62	35
B10.GD (I-A^d,I-E^o)	-	-	1	1	1	1	1	1	1	1	1	1	1
	Poly -18	10	75	56	68	68	89	83	90	47	86	63	61

T hybridoma cells were cultured in the presence of 4000R irradiated spleen cells as APC and antigen. Supernatants from 24 hr cultures were tested for their ability to stimulate [^{3}H] - thymidine incorporation by CTLL. (Refer to Materials and Methods) Results represent the mean of duplicate cultures. Standard deviations are less than 10% of the mean.

Table 4.3. Peptide antigens used in the fine antigen specificity analysis of Type B T cell hybridomas.

Peptide	Amino Acid Sequence
Poly - 18	Poly [EY**K** EYA EYA EYA EYAEYA]
	1 4 7 10 13
$(EYA)_5$	EYA EYA EYA EYA EYA
$(EYA)_5$sub5	EYA E**A**A EYA EYA EYA
$(EYA)_5$sub7	EYA EYA **A**YA EYA EYA
$(EYA)_5$sub8	EYA EYA E**A**A EYA EYA
Poly - 18	Poly [EY**K** EYA EYA EYA EYAEYA]
	1 4 7 10 13
$EYK(EYA)_4$	EY**K** EYA EYA EYA EYA
$EYK(EYA)_4$sub5	EY**K** E**A**A EYA EYA EYA
$EYK(EYA)_4$sub7	EY**K** EYA **A**YA EYA EYA
$EYK(EYA)_4$sub10	EY**K** EYA EYA **A**YA EYA
K3K or $EYK(EYA)_3EYK$	EY**K** EYA EYA EYA EY**K**

Table 4.4.

Fine antigen specificity analysis of TYPE B T cell hybridomas derived from BALB/c or (BALB/c x C57BL/6)F_1 animals.

Peptide* Antigen	Antigen Dose	Type B T cell hybridoma responses (CPM x 10^{-3})												
	µM	B4	B17	B5	B15	B9	B1	B7	B8	B16	B6	F3	F4	F1
$(EYA)_5$	10	3	1	187	175	143	168	170	131	178	177	2	163	14
	100	105	146	176	180	136	144	141	140	150	155	145	147	136
$(EYA)_5$sub5	10	1	1	1	1	16	2	37	2	1	2	119	1	4
	100	1	1	1	1	108	146	114	85	69	20	139	117	1
$(EYA)_5$sub7	10	2	2	15	2	84	131	108	166	173	110	131	23	1
	100	2	2	114	136	87	149	114	138	159	146	142	47	34
$(EYA)_5$sub8	10	1	1	1	1	1	1	1	1	1	1	145	1	1
	100	1	7	1	1	1	1	12	11	10	1	146	105	1
$EYK(EYA)_4$sub5	10	1	1	1	1	91	57	133	142	5	2	139	1	1
	100	2	2	1	140	116	156	127	126	167	128	154	111	1
$EYK(EYA)_4$sub7	10	1	157	1	1	1	75	8	63	1	1	44	44	1
	100	1	162	1	1	35	142	62	96	119	1	146	107	1
$EYK(EYA)_4$sub10	10	2	1	33	3	114	132	162	163	188	143	2	1	1
	100	1	1	159	148	109	140	134	141	169	157	135	1	92
$EYK(EYA)_3EYK$	10	1	175	3	1	83	12	80	46	144	1	138	121	1
	100	1	163	146	43	93	129	132	84	155	132	127	109	1
	-	1	1	1	1	1	1	1	1	1	1	1	1	2

T hybridoma cells were cultured in the presence of TA3 cells as APC and various antigens at two concentrations. Supernatants from 24hr cultures were tested for their ability to stimulate $[^3H]$ - thymidine incorporation by CTLL. (Refer to Materials and Methods). Results represent the mean of duplicate cultures. Standard deviations are less than 10% of the mean. Identical results were obtained in two separate experiments and data from one of the experiments is presented.

Table 4.5. Fine antigen specificity analysis of TYPE B T cell hybridomas derived from C57BL/6 --> (BALB/c x C57BL/6)F_1 chimeric animals.

Peptide Antigen	Antigen Dose µM	Type B T cell hybridoma responses (CPM x 10^{-3})							
		Ch4	Ch8	Ch3	Ch7	Ch9	Ch10	Ch2	Ch11
$(EYA)_5$	10	187	153	89	97	184	24	145	109
	100	188	163	114	152	173	145	160	130
$(EYA)_5$sub5	10	1	1	1	1	2	1	1	97
	100	32	33	1	1	2	1	59	113
$(EYA)_5$sub7	10	100	5	7	2	22	1	93	108
	100	167	119	81	126	139	65	143	114
$(EYA)_5$sub8	10	1	1	1	1	2	1	1	88
	100	1	1	1	1	1	1	1	117
$EYK(EYA)_4$sub5	10	2	2	1	1	1	1	3	155
	100	159	142	1	1	1	1	123	147
$EYK(EYA)_4$sub7	10	5	1	1	1	5	1	1	95
	100	5	1	19	6	8	1	51	130
$EYK(EYA)_4$sub10	10	149	54	12	2	35	1	113	6
	100	154	153	94	128	150	86	142	21
$EYK(EYA)_3EYK$	10	1	1	1	1	2	3	7	85
	100	144	71	70	20	94	6	121	116
-	-	2	1	2	1	1	1	1	1

Refer to Table 4.4 for details.

Table 4.6. **Analysis of allo crossreactivity patterns of Type B T cell hybridomas**

APC Spleen cells (4000R)	H-2	Antigen	Type B T cell hybridoma responses (CPM x 10^{-3})									
			B4	B17	B5	B15	B9	B1	B7	B8	B16	B6
Balb/c	d	-	1	1	1	1	1	1	1	1	1	1
C57BL/6	b	-	45	1	1	1	3	2	1	1	1	1
CBA/J	k	-	100	61	49	45	38	1	1	1	1	1
DBA/1J	q	-	1	1	1	1	13	1	1	2	1	1
B10.M/SN	f	-	101	1	1	1	1	1	1	1	1	1
B10.R III (71NS)/SN	r	-	1	1	1	1	1	1	1	1	1	1
SJL/J	s	-	1	1	1	1	1	1	1	1	1	1

			Ch4	Ch8	Ch3	Ch7	Ch9	Ch10	Ch2	Ch11	F3	F4	F1
BALB/c	d	-	1	1	1	1	1	1	1	1	1	1	1
C57BL/6	b	-	1	1	1	25	42	18	1	1	1	1	1
CBA/J	k	-	1	1	45	78	79	68	1	1	1	21	1
DBA/1J	q	-	2	1	1	3	1	1	1	25	1	1	1
B10.M/SN	f	-	1	1	1	1	1	1	16	1	1	1	1
B10.R III (71NS)/SN	r	-	1	1	1	1	1	1	1	1	1	1	1
SJL/J	s	-	1	1	1	1	1	1	1	1	1	1	1

Refer to Table 4.2 for details. Experiment was performed in the absence of exogenous peptide antigens. Identical results were obtained in two separate experiments and data from one of the experiments is presented.

Table 4.7. Summary of TYPE B (I-A^d restricted, (EYA)$_5$ specific) T cell hybridoma antigen reactivity patterns.

Type B T cell hybridomas derived from H-2^d, H-2^b-->H-2^{bxd} and H-2^{bxd} mice.

	BALB/c (H-2^d)									C57BL/6-->(BALB/c x C57BL/6)F$_1$ (H-2^b --> H-2^{bxd})									(BALB/c x C57BL/6)F$_1$ (H-2^{bxd})		
Peptides	B4	B17	B5	B15	B9	B1	B7	B8	B16	B6	Ch4	Ch8	Ch3	Ch7	Ch9	Ch10	Ch2	Ch11	F3	F4	F1
(EYA)$_5$	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
(EYA)$_5$sub5	-	-	-	-	++	++	++	++	++	+	+	+	-	-	-	-	++	++	++	++	-
(EYA)$_5$sub7	-	-	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	+
(EYA)$_5$sub8	-	+/-	-	-	-	-	+/-	+/-	+/-	-	-	-	-	-	-	-	-	++	++	++	-
EYK(EYA)$_4$sub5	-	-	-	++	++	++	++	++	++	++	++	++	-	-	-	-	++	++	++	++	-
EYK(EYA)$_4$sub7	-	++	-	-	+	++	++	++	++	-	-	-	+/-	+/-	+/-	-	++	++	++	++	-
EYK(EYA)$_4$sub10	-	-	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	++	-	++
EYK(EYA)$_3$EYK	-	++	++	+	++	++	++	++	++	++	++	++	++	+	++	+/-	++	++	++	++	-
Splenocytes																					
C57Bl/6	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+/-	-	-	-	-	-
CBA/J	++	++	+	+	+	-	-	-	-	-	-	-	+	++	++	++	-	-	-	+	-
DBA/1J	-	-	-	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
B10.M/SN	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+/-	-	-	-	-

T cell hybridoma responses: < 5000 CPM (-); 6 - 19000 CPM (+/-); 20 - 50 000 CPM (+); > 50 000 CPM(++). Data was obtained from Tables 4.4, 4.5 and 4.6 . Data from Tables 4.4 and 4.5 represents T cell hybridoma responses in the presence of 100μM antigen.

Table 4.8. Frequency of Type B T cell hybridomas derived from H-2^{d} and H-2^{b}--> H-2^{bxd} mice responding to alanine substituted (EYA)$_{5}$ and EYK(EYA)$_{4}$ peptides.

Peptides	Percent Type B T cell hybridomas responding	
	H-2^{d} derived	H-2^{b}-->H-2^{bxd} derived
(EYA)$_{5}$	100	100
(EYA)$_{5}$sub5	60	50
(EYA)$_{5}$sub7	80	100
(EYA)$_{5}$sub8	0	12
EYK(EYA)$_{4}$sub5	70	50
EYK(EYA)$_{4}$sub10	80	100

Percentage represents 10 H-2^{d} derived and 8 H-2^{b}-->H-2^{bxd} derived Type B T cell hybridomas from Table 4.7. Responses are considered positive in the >20 000 CPM range.

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CHAPTER 5

General conclusions and future prospects.

In the present study we have analyzed the T cell repertoire to the synthetic alpha-helical polypeptide Poly-18, PolyEYK(EYA)$_5$. Several aspects of the H-2^d restricted, Poly-18 specific T cell repertoire were examined. First, in normal Poly-18 responder BALB/c(H-2^d) mice we examined the hierarchy of the various Poly-18 epitopes. Second, we examined the potential of Poly-18 nonresponder C57BL/6(H-2^b) T cells to generate Poly-18 specific, I-A^d restricted, Type A and Type B T cell clonotypes after maturing in a Poly-18 responder (BALB/c x C57BL/6)F1 (H-2^{bxd}) environment. Finally, we examined the heterogeneity of the Poly-18 specific T cell response directed at (EYA)$_5$, one of the Poly-18 immunodominant epitopes, in normal BALB/c(H-2^d) as well as chimeric C57BL/6(H-2^b)->(BALB/c x C57BL/6)F1 animals. The findings of our study are summarized and discussed below.

Hierarchy of antigenic determinants in the synthetic polypeptide antigen Poly-18

1. The repeating nature of the Poly-18 antigen has enabled us to synthesize all the possible 12 and 15 amino acid long sequential overlapping epitopes of Poly-18. Since Poly-18 and its fragments do not need to be processed prior to being presented to Poly-18 specific T cells, we used the synthetic peptides representing the sequential Poly-18 epitopes to identify immunodominant epitopes of the Poly-18 antigen and analyse factors important in determining epitope dominance. Our analysis indicates that Poly-18 has 2

immunodominant epitopes, $(EYA)_4$ [or $(EYA)_5$] and $EYK(EYA)_3$ [or $EYK(EYA)_4$] and that immunodominance is strictly correlated with epitope immunogenicity as well as epitope antigenicity. It is interesting to note that although all of the Poly-18 epitopes have considerable amino acid sequence overlap, the clones which are elicited by the 2 immunodominant epitopes do not crossreact on the remaining Poly-18 epitopes. The epitope $(EYA)_3EYK$ appears to be subdominant since it can elicit Poly-18 specific T cells when it is used as the immunogen but does not appear to be part of the Poly-18 repertoire when Poly-18 is used as the immunogen. Similar observations have been made in other antigenic systems such as sperm whale myoglobin [1] and hen egg lysozyme [2,3].

2. Our analysis of the previously described Type A and Type B Poly-18 specific T cell clonotypes in BALB/c mice [4] using Poly-18 specific T cell hybridomas indicates that the Type B clonotype, which can be elicited by the lysine free peptides $(EYA)_4$ and $(EYA)_5$, is very heterogeneous and much larger that the Type A clonotype, which can be elicited by the lysine containing peptides $EYK(EYA)_3$ and $EYK(EYA)_4$, and that this is not related to the availability of the lysine containing epitope $EYK(EYA)_4$.

3. Our analysis also indicates that peptides representing the two immunodominant Poly-18 epitopes elicit a broader range of clones than the whole Poly-18 molecule since using Poly-18 derived peptides as immunogens elicits T cell phenotypes which are not elicited when native Poly-18 is used as an immunogen. Whether these differences are related to antigen processing is not clear since Poly-18 and its related peptides do not appear to require antigen processing prior to presentation to Poly-18 specific T cell hybridomas [5]. However Fox et al.[6] have shown that the further processing of a minimal cytochrome c peptide which did not require to be processed for recognition by

cytochrome specific T cells altered its interaction with MHC molecules.

Analysis of Poly-18 specific T cell clonotypes in normal Responder and Nonresponder->(Responder x Nonresponder)F1 bone marrow radiation chimeric mice

1. Responsiveness to Poly-18 is under Ir-gene control. Mice of the H-2^d haplotype ie. BALB/c are responders and mice of the H-2^b haplotype ie. C57BL/6 are nonresponders. Responsiveness is dominant over nonresponsiveness and heterozygous H-2^{bxd} mice ie. (BALB/c x C57BL/6)F1 are also Poly-18 responders [7]. We demonstrate that T cell precursors from Poly-18 nonresponder H-2^b mice can be educated in a responder H-2^{bxd} environment to recognize Poly-18, an antigen under Ir-gene control, in the context of the responder H-2^d I-region gene products.

2. However, our analysis indicates that the Poly-18 specific T cell population in the C57BL/6(H-2^b)->(BALB/c x C57BL/6)F1 [H-2^b->F1] chimeras is phenotypically different from the Poly-18 specific T cell populations in normal BALB/c, (BALB/c x C57BL/6)F1 and chimeric (BALB/c x C57BL/6)F1->(BALB/c x C57BL/6)F1 animals. The H-2^b->F1chimeras do not appear to generate clones which respond strongly to the lysine containing peptide K3K, although they generate large numbers of clones which respond strongly to the lysine free peptide $(EYA)_5$.

3. We find that our H-2^b->F1 chimeras are immunocompetent even though their unseparated lymph node cell populations may contain undetectable levels of host derived cells. The expansion of H-2^d restricted, Poly-18 specific, T cell clones in the H-2^b->F1 chimeras must be due to some long lived radio resistant $(H2^{bxd})$F1 Ia positive antigen presenting cells since the Poly-18 specific

T cell hybridomas generated from these chimeric mice are I-A^{d} restricted and do not concomitantly respond to Poly-18 in the context of H-2^{b} MHC gene products. Furthermore, Poly-18 specific T cell lines generated from these H-2^{b}->F1 chimeras do not respond to H-2^{d} or H-2^{b} splenic antigen presenting cells alone. Other investigators working in Ir gene controlled antigen systems have found it necessary to infuse responder APC at the time of antigen challenge to obtain antigen specific responses restricted to the responder MHC gene products [8,9]. Although we did not find it necessary to do so, we can not exclude the possibility that the limiting numbers of (H2^{bxd})F1 APC in the H-2^{b}->F1 chimeras could be responsible for the phenotypic differences observed between the BALB/c and H-2^{b}->F1 Poly-18 specific T cell repertoires. Alternatively, some T cell repertoire deletion caused by regulatory or tolerance mechanisms may be the primary cause of this epitope specific nonresponsiveness.

The Poly-18 epitope $(EYA)_5$ is the focus of a highly polyclonal T cell response

1. Our analysis of the diversity of the I-A^{d} restricted T cell response directed at the Poly-18 immunodominant epitope $(EYA)_5$ indicates that the response is extremely heterogeneous in normal BALB/c(H-2^{d}) mice. Using a panel of alanine and/or lysine substituted $(EYA)_5$ peptides to analyze the peptide specific responses and a panel of haplotype variant splenocytes in the absence of exogenous peptide antigens to analyze the allo crossreactivity patterns of 10 randomly chosen I-A^{d} restricted $(EYA)_5$ specific (Type B) T cell hybridomas we could identify 7 unique T cell phenotypes. This is further supported by the demonstration that at least 7 of these Type B T cell

hybridomas use unique TcR VαVβ gene pair combinations (P. Kilgannon et al. manuscript submitted).

2. Although we only analyzed 3 Type B T cell hybridomas derived from (BALB/c x C57BL/6)F1 animals, the $(EYA)_5$ specific T cell response in these animals is also likely to be very heterogenous since all 3 of these hybridomas express a unique T cell phenotype.

3. Our fine antigen specificity analysis of 8 randomly chosen Type B T cell hybridomas derived from $H\text{-}2^b$->F1 chimeric animals indicates that although this Type B T cell population is not as heterogeneous as the BALB/c derived Type B T cell population, it does represent at least 4 unique T cell phenotypes.

4. Although no particular Type B phenotype was found to dominate in the three Poly-18 responder $H\text{-}2^d$, $H\text{-}2^{bxd}$ and $H\text{-}2^b$->$H\text{-}2^{bxd}$ animals, the T cell repertoires of $H\text{-}2^d$ and $H\text{-}2^b$->$H\text{-}2^{bxd}$ animals exhibit some similarities. First, both of these Type B T cell repertoires contain a large proportion ie. 50% of T cell clones which exhibit allocrossreactivity on CBA/J($H\text{-}2^k$) spleen cells. Analysis of Poly-18 specific T cell lines suggests that this reactivity may be due to the recognition of $Mls\text{-}1^a$ encoded molecules presented in the context of $H\text{-}2^k$ MHC gene products. This is further supported by our observation that 8 of the 10 CBA/J allocrossreactive Type B T cell hybridomas use the TcR Vβ6 gene (P. Kilgannon et al. manuscript submitted and unpublished data), which has been shown by MacDonald et al.[10] to correlate with $Mls\text{-}1^a$ recognition in various $H\text{-}2^k$ and $H\text{-}2^d$ mouse strains. Second, almost all Type B T cell hybridomas from $H\text{-}2^d$ as well as $H\text{-}2^b$->$H\text{-}2^{bxd}$ animals fail to respond to one of the $(EYA)_5$ derived peptides, namely $(EYA)_5$sub8. The tyrosine residue at position 8 of the $(EYA)_5$ peptide therefore appears to be a very critical residue for almost all $(EYA)_5$ specific T cell clones. Such specific recognition of a few selected critical residues may be the only common feature shared by this diverse T cell

population.

5. In concordance with our earlier observation that H-2^b->H-2^{bxd} animals fail to generate Poly-18 specific T cell clones with strong K3K reactivity, we find that only one of the Type B T cell hybridomas derived from H-2^b->H-2^{bxd} animals is strongly K3K reactive while half of the BALB/c derived Type B T cell hybridomas are strongly K3K reactive. As discussed earlier, it is not clear whether the limiting numbers of responder APC in the H-2^b->H-2^{bxd} chimeras or other negative or regulatory influences are responsible for the absence of strongly K3K reactive T cell clones in their Poly-18 specific T cell repertoires, however the absence of these clones could be contributing to the apparently decreased heterogeneity of the Type B T cell population derived from these animals.

Future prospects

Several aspects of the Poly-18 specific T cell repertoire can be explored in future analyses. Since we have shown that Poly-18 epitopes presented in the context of peptide fragments versus the Poly-18 alpha helix elicit different Poly-18 specific T cell clones, the question of processing requirements of various minimum Poly-18 peptides can be explored. Our initial studies analyzing the secondary structure of the 15 amino acid minimum peptides using circular dichroism and NMR analyses indicates that there is little or no secondary structure in these peptide antigens, although they do assume partial alpha-helical structure in non aqueous environments (M.Boyer et al. unpuplished data). Moreover, no correlation could be drawn regarding the structure-function relationship in these peptides. However, the Poly-18 molecule normally assumes an alpha-helical conformation. Making peptides of increasing length and thereby increasing their potential to generate an alpha helix could provide us with a means of studying the influence of peptide structure on clonotype selection.

We can also explore the possible influence of background genes on the selection of Poly-18 specific T cell clonotypes. Antigen specificity and TcR Vβ gene usage analysis can be performed in H-2^d bearing mouse strains with variable background genes such as B10.GD, B10.D2, DBA/2J, C3H.OH and NZB. We can also analyze the influence of other MHC genes in conjuction with background genes by analyzing various F1 animals expressing H-2^d MHC molecules. In particular we could analyze the Poly-18 specific T cell response in animals such as (BALB/c x CBA/1J)F1(H-2^{kxd}) or (BALB/c x DBA/2J)(H-2^d) as these animals express the Mls-1^a antigen in the context of H-2^k or H-2^d MHC molecules and would therefore not be expected to use the dominant TcR Vβ6 gene in response to the Poly-18 antigen. It would also be possible to use Poly-18 transgenic mice and analyze the effect of in vivo Poly-l8 expression in soluble and/or membrane bound form on the expressed antigen specific T cell repertoire.

We can also explore the capacity of various nonresponder haplotypes to learn intrathymically to recognize Poly-18 in the context of H-2^d MHC gene products by making various nonresponder->responder chimeras. The results may be quite variable with different nonresponder strains as they appear to differ normally in their capacity to respond to various Poly-18 related antigens. For instance, both H-2^b and H-2^k strains of mice are nonresponders to Poly-18, however, only the H-2^k strain is also a nonresponder to the closely related antigen Poly-EYA, which is simply Poly-18 with all of its lysine residues substituted by alanine residues. We could therefore compare the gene usage of H-2^b restricted, $(EYA)_5$ specific, T cell clones versus H-2^d restricted, $(EYA)_5$

specific, T cell clones. Since we know that in the H-2^d mice this response is dominated by T cells using the TcR Vβ6 gene, it would be interesting to determine what TcR Vβ gene dominates, if any, in the H-2^b restricted T cell response. Since the antigen is identical in both cases, the use of particular TcR Vβ genes would have to be a direct consequence of the MHC gene products expressed.

In conclusion, the Poly-18 antigen system, which we presumed to have fewer potential epitopes and consequently a less heterogeneous T cell repertoire, proved to be remarkably complex. The diversity of the T cell response generated to this rather homogeneous antigen provides us with a glimpse of the nature and complexity of the immune system.

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